UNIVERSIDAD DE GRANADA
FACULTAD DE CIENCIAS
DEPARTAMENTO DE FISIOLOGÍA VEGETAL

PROGRAMA DE DOCTORADO: BIOLOGÍA FUNDAMENTAL Y DE SISTEMAS

UNIVERSIDAD DE GRANADA

Eficacia de plantas mutantes TILLING de *Brassica rapa* (BraAcax1.a) en la biofortificación con Ca^{2+} y en la resistencia a distintos estreses abióticos

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Tesis Doctoral
Granada, Noviembre 2021

Editor: Universidad de Granada. Tesis Doctorales
Autor: Eloy Navarro León
ISBN: 978-84-1117-088-8
URI: http://hdl.handle.net/10481/71397

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Memoria de tesis doctoral presentada por el licenciado en biología Eloy Navarro León para optar al grado de Doctor en Biología Fundamental y de Sistemas con mención internacional.

El Doctorando

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Granada, Noviembre 2021

Financiación y publicaciones

El trabajo que se presenta en esta memoria de Tesis Doctoral ha sido realizado en el Grupo de Investigación “Fisiología y fitotecnia de cultivos para el desarrollo de una agricultura sostenible” (AGR-282, Plan Andaluz de Investigación, Junta de Andalucía), del Departamento de Fisiología Vegetal de la Facultad de Ciencias de la Universidad de Granada (España). Este trabajo ha sido financiado por dicho grupo.

La realización de la presente Tesis doctoral fue financiada por las siguientes fuentes:

- Beca de Formación de Profesorado Universitario (Ref. FPU14/01858). Desde el 30 de Octubre de 2015 al 30 de Octubre de 2021
- Beca de movilidad internacional de estudiantes de Programas de doctorado universidad de granada realizada en Dipartimento di Biologia, Università degli Studi di Napoli Federico II (Nápoles, Italia). Bajo la supervisión del Dr. Sergio Esposito durante el 01 de Septiembre de 2018 al 30 de Noviembre del 2018.

Los artículos presentados en esta memoria de tesis doctoral han sido publicados en revistas internacionales:

- **Navarro-León, E.**, Ruiz, J. M., Graham, N., & Blasco, B. (2018). Physiological profile of CAX1a TILLING mutants of *Brassica rapa* exposed to different calcium doses. *Plant Science*, *272*, 164-172. https://doi.org/10.1016/j.plantsci.2018.04.019

- **Navarro-León, E.**, Ruiz, J. M., Albacete, A., & Blasco, B. (2019). Effect of CAX1a TILLING mutations and calcium concentration on some primary metabolism processes in *Brassica rapa* plants. *Journal of plant physiology*, *237*, 51-60. https://doi.org/10.1016/j.jplph.2019.04.002

- **Navarro-León, E.**, Ruiz, J. M., Albacete, A., & Blasco, B. (2020). Tolerance to cadmium toxicity and phytoremediation potential of three *Brassica rapa* CAX1a TILLING mutants. *Ecotoxicology and Environmental Safety*, *189*, 109961. https://doi.org/10.1016/j.ecoenv.2019.109961

- **Navarro-León, E.**, López-Moreno, F. J., de la Torre-González, A., Ruiz, J. M., Esposito, S., & Blasco, B. (2020). Study of salt-stress tolerance and defensive mechanisms in *Brassica rapa* CAX1a TILLING mutants. *Environmental and Experimental Botany*, *175*, 104061. https://doi.org/10.1016/j.envexpbot.2020.104061

- **Navarro-León, E.**, López-Moreno, F. J., Atero-Calvo, S., Albacete, A., Ruiz, J. M., & Blasco, B. (2020). CAX1a TILLING Mutations modify the hormonal balance controlling growth and ion homeostasis in *Brassica rapa* plants subjected to salinity. *Agronomy*, *10*(11), 1699. https://doi.org/10.3390/agronomy10111699

- **Navarro-León, E.**, Paradisone, V., López-Moreno, F. J., Rios, J. J., Esposito, S. & Blasco B. Effect of CAX1a TILLING mutations on photosynthesis

performance in salt-stressed *Brassica rapa* plants. *Plant Science*, *311*, 111013. https://doi.org/10.1016/j.plantsci.2021.111013

Parte de los resultados obtenidos ha sido presentados en los siguientes congresos y reuniones científicas:

- XXII Reunión de la Sociedad Española de Fisiología Vegetal y XV Congreso Hispano-Portugués de Fisiología Vegetal. Barcelona (26/06/2017 – 29/06/2017). Póster y Flash talk: Evaluation of three CAX1 *Brassica rapa* mutants grown under different Ca doses
- Innovating Plant Nutrition. XVII Simpósio Luso-Espanhol de Nutrição Mineral das Plantas. Portugal (25/07/2018 – 28/07/2018). Presentación oral: *Brassica rapa* CAX1a TILLING mutant as a potential tool for Cd phytoremediation
- II Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias. Almería. Póster: *BraA.cax1a-12* y *BraA.hma4a-3*: dos mutantes TILLING de *Brassica rapa* con potencial para la biofortificación de elementos minerales
- XXIV Reunión de la Sociedad Española de Fisiología Vegetal y XVII Congreso Hispano-Portugués de Biología de Plantas. Vigo (07/07/2021 – 09/07/2021). Póster: A variation in vacuolar calcium transporter CAX1 generated by TILLING provides higher tolerance to salinity in *Brassica rapa* plants

ÍNDICE

ABREVIATURAS

Φ_{Eo}	Producto cuántico máximo de la fotoquímica primaria
Ψ_{Eo}	Eficiencia en el transporte de electrones
A	Tasa fotosintética neta
AAs	Aminoácidos
AAT	Aspartato amino transferasa
ABA	Ácido abscísico
ABS	Flujo de fotones absorbido por los pigmentos antena
ACC	Ácido 1-aminociclopropano-1-carboxílico
AIA	Ácido indolacético
APX	Ascorbato peroxidasa
Arg	Arginina
AS	Selectividad de absorción
AsA	Ascorbato
Asp	Ácido aspártico
CAT	Catalasa
CaUpE	Eficiencia en la absorción de Ca
CaUtE	Eficiencia en la utilización de Ca
CAX	Cation/H^+ exchanger
Chl	Clorofila
Ci	CO_2 intercelular
CKs	Citoquininas
CS	Citrato sintasa
DC	Coeficiente de distribución
DHA	Dehidroascorbato
DIo	Energía de disipación
E	Tasa de transpiración
EC	Perdida de electrolitos
EMS	Etilmetanosulfonato
ETto	Transporte de electrones
Fm	Fluorescencia máxima
Fo	Fluorescencia inicial
FS	Fotosíntesis

FSII	Fotosistema II
Fv	Fluorescencia variable
G6PDH	glucosa-6-fosfato deshidrogenasa
GA	Giberelina
GDH	Glutamato deshidrogenasa
GGAT	glutamato: glioxilato aminotransferasa
Glu	Ácido glutámico
Gly	Glicina
GO	Glioxilato oxidasa
GOGAT	Glutamato sintasa
GPX	Glutation peróxidasa
GR	Glutation reductasa
GS	Glutamina sintetasa
gs	Conductancia estomática
GSH	Glutation
GSSG	Glutation oxidado
His	Histidina
HSP	Proteinas de choque térmico
HOR	Fitohormonas
Ile	Isoleucina
iP	Isopentenil adenina
IRGA	Analizador de gases por infrarojos
LOX	Lipoxigenasa
MDA	Malondialdehído
MDH	Malato deshidrogenasa
NBTs	Nuevas tecnologías de breeding
NR	Nitrato reductasa
OAs	Ácidos orgánicos
OEC	Complejo productor de oxígeno fotosintético
OMG	Organismos modificados genéticamente
OPPP	Via oxidativa de las pentosas fosfato
PAR	Radiacion fotosinteticamente activa
PCA	Análisis de componentes principales
PCs	Fitoquelatinas
PEPC	Fosfoenolpiruvato carboxilasa
PI_{ABS}	Indice de funcionamiento fotosintético

Pro	Prolina
PS	Fotosistema
PSR	Genes de respuesta al fósforo
PUE	Eficiencia en el uso del fósforo
RC	Centro de reacción
ROS	Especies reactivas de oxígeno
Rubisco	Ribulosa-1,5-bisfosfato carboxilasa/oxigenasa
Ser	Serina
SOD	Superóxido dismutasa
STI	Índice de tolerancia a la salinidad
t Fm	Tiempo hasta alcanzar Fm
TCA	Ciclo de los ácidos tricarboxílicos
Thr	Treonina
TILLING	Targeting induced local lesions in genomes
TRo	Energía de excitación atrapada
TS	Selectividad de traslocación
tZ	trans-zeatina
Val	Valina
WUE	Eficiencia en el uso del agua

RESUMEN / ABSTRACT

La agricultura debe aumentar considerablemente su producción para poder alimentar a la creciente población mundial y con la dificultad añadida del actual escenario de cambio climático. Por otro lado, no solo es necesario proveer alimentos en cantidad suficiente, también es importante que estos alimentos sean seguros, nutritivos y de calidad. La biotecnología se postula coma una herramienta esencial para superar estos desafíos mediante el desarrollo de variedades más productivas, adaptadas a condiciones adversas, que requieran menor cantidad de insumos y que aumenten la acumulación de elementos minerales esenciales. El TILLING (Targeting Induced Local Lesions in Genomes) es una técnica que ha demostrado ser útil en la mejora de los cultivos y que presenta la capacidad de identificar mutantes sin conocimiento previo del fenotipo y no requiere la obtención de transgénicos.

Uno de los objetivos para la mejora vegetal podría ser la generación de variedades con una homeostasis más eficiente del calcio (Ca) debido a que este elemento es un macronutriente esencial y desempeña una función crucial en la señalización celular de las plantas. Este objetivo se podría alcanzar mediante la modificación de uno de los principales transportadores de Ca^{2+} de las plantas, los CAX (CAtion eXchangers). Estos transportadores son claves en la generación de distintos perfiles de Ca^{2+} citosólicos que sirven para responder a las señales ambientales o en los procesos de señalización celulares. Hay diversos transportadores CAX, pero CAX1 es el que posee una mayor actividad de antiporte H^+/Ca^{2+} vacuolar. Diversos experimentos han conseguido mejorar la acumulación de Ca en plantas mediante la manipulación de este transportador.

Debido a la importancia fisiológica del Ca y de CAX1, la modificación de este transportador puede afectar a los distintos procesos metabólicos de la planta entre los que destacan: la fotosíntesis, los procesos de estrés oxidativo, el metabolismo del nitrógeno (N), el ciclo de los ácidos tricarboxilicos (TCA) y la regulación fitohormonal. Asimismo, se ha postulado que la manipulación de los niveles de expresión de los transportadores CAX puede tener un efecto positivo en la productividad de la planta y/o la calidad nutricional de los alimentos. Por otro lado, ciertas modificaciones en estos transportadores pueden cambiar la especificidad de sustrato y por tanto las plantas resultantes pueden ser útiles para la fitorremediación de suelos contaminados con metales pesados. Además, los transportadores CAX parecen jugar un papel esencial en las respuestas de las plantas ante un estrés. Así, la inducción de los transportadores CAX produciría un aumento en la concentración de Ca^{2+} en las plantas lo que reduciría el efecto negativo de estreses abióticos tales como la salinidad y la presencia de metales pesados.

Dado el potencial para la mejora vegetal de modificaciones en CAX1, se ha usado la técnica TILLING para generar distintos mutantes con cambios aminoácidos en este transportador en una especie modelo en investigación como es *Brassica rapa* ssp. trilocularis 'R-o-18'. Para esta Tesis se han seleccionado tres de estos mutantes que poseen un único cambio aminoacídico distinto cada uno (*BraA.cax1a-4*, *BraA.cax1a-7* y *BraA.cax1a-12*). La realización de la Tesis estuvo justificada por la necesidad de comprobar la efectividad del TILLING en la mejora vegetal en agricultura, para profundizar en el conocimiento existente sobre la función de CAX1 en las plantas y por la potencial aplicabilidad

de los resultados obtenidos a cultivos próximos a *B. rapa* de gran interés agronómico. **Así, el objetivo general de la presente tesis doctoral es el estudio de los tres mutantes *BraA.cax1a* centrado en la evaluación bioquímica y fisiológica con el fin de definir la función y papel del gen CAX1 en la mejora del cultivo de *B. rapa*.** En concreto se analizó el efecto de dichas mutaciones en el transportador CAX1 de *B. rapa* en plantas cultivadas bajo tres condiciones: diferentes concentraciones de Ca en el medio de cultivo, toxicidad por cadmio (Cd) y estrés por salinidad.

El **primer objetivo** de esta Tesis doctoral fue comprobar cómo se producía la acumulación de Ca^{2+} en las plantas mutantes crecidas a diferentes concentraciones de este catión en el medio de cultivo y evaluar la calidad nutricional y los efectos fisiológicos de cada mutación en CAX1 en la biofortificación con este elemento. Los resultados mostraron que los distintos mutantes *BraA.cax1a* presentaron diferente respuesta ante cambios en la disponibilidad de Ca en el medio en cuanto a su crecimiento, su fisiología y su acumulación de nutrientes. Las distintas mutaciones no causaron una mayor acumulación de Ca ante condiciones deficientes en el medio, pero si aumentaron su acumulación con un aporte óptimo y tóxico de este catión. Cabe destacar que la línea *BraA.cax1a-12* acumuló más Ca que el parental R-o-18 en condiciones control, pero limitó su acumulación ante elevadas concentraciones evitando así su toxicidad. En cuanto a otros elementos minerales, los mutantes empleados en esta Tesis acumularon más Mg en las hojas cuando el Ca se aportó en una concentración adecuada y acumularon más Fe independientemente de la dosis de Ca.

Con respecto a los efectos fisiológicos, se observó que las mutaciones *BraA.cax1a* afectaron claramente a la fisiología y al metabolismo de las plantas. Así, *BraA.cax1a-4* y *BraA.cax1a-7* mostraron síntomas de estrés como una menor cantidad de clorofila (Chl), una alteración del rendimiento de la fotosíntesis y mayores niveles de especies reactivas de oxígeno (ROS). La mutación *BraA.cax1a-7* redujo la actividad de algunas enzimas del metabolismo del nitrógeno (N) y esta mutación junto con la mutación *BraA.cax1a-4* aumentó la actividad de las enzimas de la fotorrespiración analizadas. Sin embargo, la mutación *BraA.cax1a-12* no afectó negativamente al metabolismo de la planta y proporcionó una mejor tolerancia a las dosis altas de Ca. Esta tolerancia podría ser proporcionada por la mejora de las actividades de las enzimas del metabolismo del N, de las actividades fosfoenolpiruvato carboxilasa (PEPC) y citrato sintasa (CS), y por una concentración más elevada de glutamato (Glu) y malato. La acumulación más alta de ácido indolacético (AIA) y la menor presencia del precursor de etileno también podría contribuir a la mayor tolerancia de *BraA.cax1a-12*.

El **segundo objetivo** de esta Tesis Doctoral fue comprobar la tolerancia de cada mutante *BraA.cax1a* frente a la **toxicidad por Cd** y detectar procesos clave que determinen una mayor tolerancia a cada tipo de estrés. Con respecto a este objetivo se constató que la mutación *BraA.cax1a-7* y especialmente la mutación *BraA.cax1a-12* proporcionaron una acumulación más elevada de Cd en las hojas con respecto a R-o-18. Así, *BraA.cax1a-12* registró la capacidad más alta de fitoextracción con más del triple del contenido de Cd de R-o-18. A pesar de ello,

BraA.cax1a-12 presentó una mejor tolerancia a la toxicidad del Cd que podría deberse a una mejor homeostasis del Ca^{2+} en condiciones de estrés por este metal y a la mayor acumulación de Mg y Ca. Igualmente, dichas plantas presentaron una adecuada eficiencia fotosintética ante condiciones de toxicidad de Cd. Por otro lado, se observó que el genotipo *BraA.cax1a-4* presentó una respuesta similar a la de las plantas R-o-18 aunque tuvo una capacidad más eficiente de detoxificación de H_2O_2 a través del ciclo ascorbato/glutation (AsA/GSH). Sin embargo, *BraA.cax1a-12* presentó la más alta actividad del ciclo de AsA/GSH y una mejor capacidad de detoxificación de ROS. Además este genotipo presentó una actividad más elevada del ciclo de los ácidos tricarboxílicos (TCA) y una mayor acumulación de malato. Por otro lado, el genotipo *BraA.cax1a-7* registró una respuesta negativa porque mostró menores concentraciones de AsA y ácidos orgánicos (OAs) y una actividad inferior del TCA. Además, los niveles más altos de giberelina 4 (GA4), y la menor presencia del precursor del etileno ácido 1-aminociclopropano-1-carboxílico (ACC) en plantas *BraA.cax1a-12* también podrían contribuir a su mayor tolerancia frente al Cd.

El segundo tipo de estrés analizado en la presente Tesis Doctoral fue el **estrés por salinidad**. Los resultados mostraron que las distintas mutaciones *BraA.cax1a* afectaron a la respuesta frente a la salinidad. Así, la mutación *BraA.cax1a-4* proporcionó una mejor tolerancia a la presencia de NaCl en el medio de cultivo. Esta tolerancia podría ser inducida por un mayor contenido de Ca y K en la parte aérea, una detoxificación más eficiente de ROS a través de las enzimas superóxido dismutasa (SOD) y catalasa (CAT), un mejor estado

redox del AsA y una mayor actividad glucosa-6-fosfato deshidrogenasa (G6PDH). Además, la mutación B*raA.cax1a-4* proporcionó una mejora en el rendimiento fotosintético que se manifestó en unos valores más altos de eficiencia en el uso del agua (WUE), del ratio entre fluorescencia variable y fluorescencia máxima (Fv/Fm), de flujos de electrones y una mayor acumulación de ribulosa-1,5-bisfosfato carboxilasa/oxigenasa (Rubisco). En contraste, la mutación *BraA.cax1a-7* produjo algunos efectos negativos en el rendimiento de la fotosíntesis, como por ejemplo elevados valores emisión de fluorescencia y menores acumulaciones de Rubisco y carbohidratos solubles y una actividad G6PDH más baja. En cuanto al perfil fitohormonal, *BraA.cax1a-4* mostró una concentración más alta de fitohormonas que podría estar relacionada con su mejor tolerancia a la salinidad. Concretamente, AIA y giberelinas (GAs) podrían promover el crecimiento en condiciones salinas y el ácido abscísico (ABA) podría dar lugar a una mejor respuesta frente al estrés y a una mayor eficiencia en el uso del agua.

Como **conclusión** general, en esta tesis se describieron los cambios fisiológicos en *B. rapa* producidos por distintas mutaciones en el transportador CAX1 y su respuesta a distintos estreses. Se identificó a la mutación *BraA.cax1a-12* como útil para la biofortificación de Ca, Mg, y Fe y para la fitorremediación de Cd y a la mutación *BraA.cax1a-4* para proporcionar una mayor tolerancia a la salinidad. Además, se identificaron procesos metabólicos claves en la tolerancia a cada tipo de estrés estudiado.

ABSTRACT

Agriculture must increase its production considerably to feed the world's growing population in the framework of the current climate change scenario. On the other hand, it is not just necessary to provide food in sufficient quantity, but it is also important that this food be safe, nutritious, and high quality. Biotechnology is an essential tool for overcoming these challenges by developing more productive varieties that are adapted to adverse conditions, require fewer inputs, and increase the accumulation of essential mineral elements. TILLING (Targeting Induced Local Lesions in Genomes) is a technique that has proven to be useful in crop improvement and can identify mutants without prior knowledge of the phenotype and does not require transgenic breeding.

One of the objectives for plant breeding could be the generation of varieties with more efficient calcium (Ca) homeostasis because this element is an essential macronutrient and plays a crucial role in plant cell signaling. This objective could be achieved by modifying one of the main Ca^{2+} transporters in plants such as CAX (CAtion eXchangers). These transporters are key in the generation of distinct Ca^{2+} cytosolic profiles that serve to respond to environmental signals or in cell signaling processes. There are several CAX transporters, but CAX1 has the highest vacuolar H^{+}/Ca^{2+} antiporter activity. Several experiments have succeeded in improving Ca accumulation in plants by manipulating this transporter.

Because of the physiological importance of Ca and CAX1, the modification of this transporter can affect different metabolic processes in the plant, including photosynthesis, oxidative stress processes, nitrogen (N) metabolism, the tricarboxylic acid (TCA) cycle, and phytohormonal regulation. It has also been postulated that manipulation of the CAX expression levels could have a positive effect on plant productivity and/or the nutritional quality of food. On the other hand, certain modifications in these transporters may change substrate specificity and thus the resulting plants may be useful for phytoremediation of heavy metal contaminated soils. Moreover, CAX transporters play an essential role in plant stress responses. Thus, the induction of CAX transporters would produce an increase in the Ca^{2+} concentration in plants which would reduce the negative effect of abiotic stresses such as salinity and the presence of heavy metals.

Given the potential for plant breeding of CAX1 modifications, the TILLING technique has been used to generate different mutants with amino acid changes in this transporter in a model species in research such as *Brassica rapa* ssp. trilocularis 'R-o-18'. Three of these mutants were selected for this Thesis, each possessing a single distinct amino acid change (*BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12*). The proposal of this Thesis was justified by the need to test the effectiveness of TILLING in plant breeding in agriculture, to deepen the existing knowledge on CAX1 function in plants, and by the potential applicability of the obtained results to crops of great agronomic interest phylogenetically close to *B. rapa*. Thus, **the general objective of this Thesis is the study of the three *BraA.cax1a* mutants focused on the biochemical and physiological evaluation to define the function and role of the CAX1 gene in the**

improvement of *B. rapa*. Specifically, the effect of these mutations in the CAX1 transporter of *B. rapa* was analyzed in plants grown under three conditions: different Ca concentrations in the culture medium, cadmium (Cd) toxicity, and salinity stress.

The **first objective** of this PhD Thesis was to analyzed Ca^{2+} accumulation in the mutant plants grown at different concentrations of this cation in the culture medium and to evaluate the nutritional quality and physiological effects of each mutation in CAX1 in biofortification with this element. The results showed that the different *BraA.cax1a* mutants presented different responses to changes in Ca availability in the medium in terms of growth, physiology, and nutrient accumulation. The different mutations did not cause an increased Ca accumulation under deficient conditions in the medium, but they did increase its accumulation under optimal and toxic Ca supply. It should be noted that the *BraA.cax1a-12* line accumulated more Ca than the parental R-o-18 under control conditions, but limited its accumulation under high concentrations, thus avoiding its toxicity. Considering other mineral elements, the mutants used in this Thesis accumulated more Mg in the leaves when Ca was supplied at an adequate concentration and accumulated more Fe regardless of the applied Ca dose.

The analysis of physiological effects showed that *BraA.cax1a* mutations clearly affected plant physiology and metabolism. Thus, *BraA.cax1a-4* and *BraA.cax1a-7* plants showed stress symptoms such as lower chlorophyll (Chl), altered photosynthetic performance, and higher levels of reactive oxygen species

(ROS). The *BraA.cax1a-7* mutation reduced the activity of some nitrogen (N) metabolism enzymes and this mutation and the *BraA.cax1a-4* mutation increased the activity of the analyzed photorespiration enzymes. However, the *BraA.cax1a-12* mutation did not negatively affect plant metabolism and provided improved tolerance to high Ca doses. This tolerance could be provided by enhanced activities of N-metabolism enzymes, phosphoenolpyruvate carboxylase (PEPC) and citrate synthase (CS) activities, and higher glutamate (Glu) and malate concentration. The higher accumulation of indoleacetic acid (IAA) and the lower presence of the ethylene precursor could also contribute to the higher tolerance of *BraA.cax1a-12* plants.

The **second objective** of this PhD Thesis was to test the tolerance of each *BraA.cax1a* mutant to **Cd toxicity** and to detect key processes that determine a higher tolerance to each type of stress. It was found that the *BraA.cax1a-7* mutation and especially the *BraA.cax1a-12* mutation provided a higher accumulation of Cd in leaves in comparison to R-o-18 plants. Thus, *BraA.cax1a-12* reached the highest phytoextraction capacity with more than three times the Cd content of R-o-18. Nevertheless, *BraA.cax1a-12* showed better tolerance to Cd toxicity, which could be due to enhanced Ca^{2+} homeostasis under Cd stress conditions and to the higher accumulation of Mg and Ca. Likewise, these plants showed an adequate photosynthetic efficiency under Cd toxicity conditions. On the other hand, it was observed that the *BraA.cax1a-4* genotype showed a similar response to that of R-o-18 plants, although it had a more efficient H_2O_2 detoxification capacity through the ascorbate/glutathione (AsA/GSH) cycle. However, *BraA.cax1a-12* presented the highest AsA/GSH cycle activity and

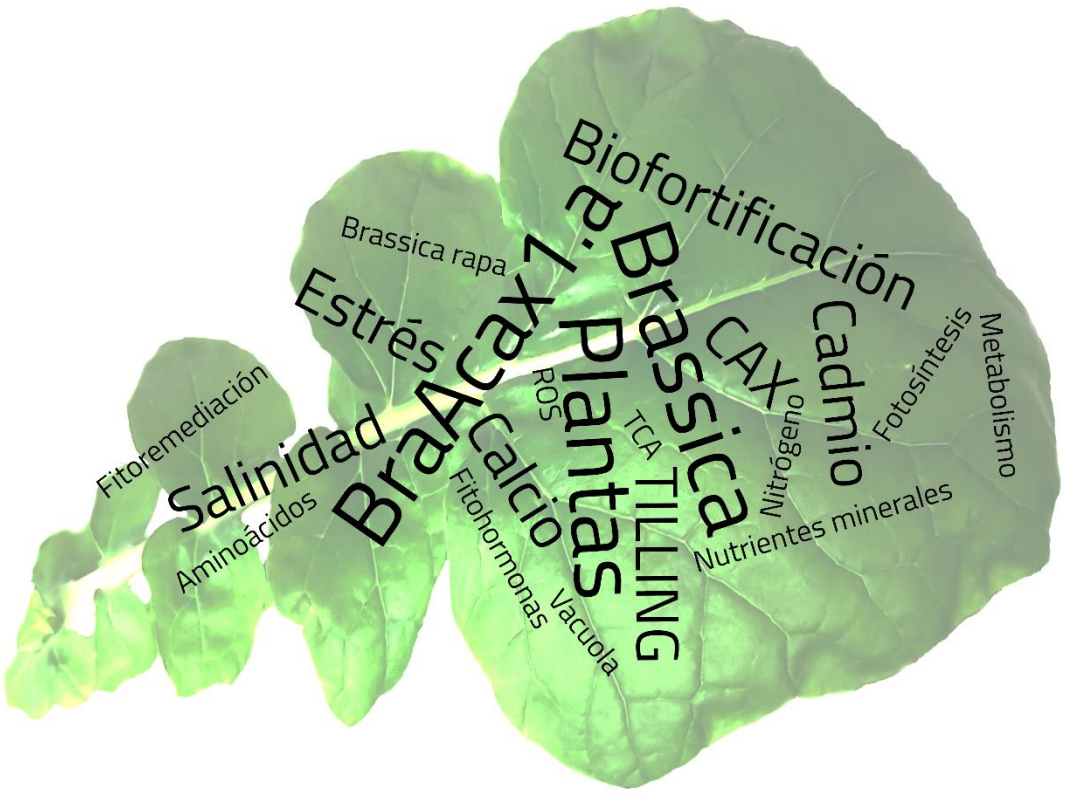
better ROS detoxification capacity. In addition, this genotype showed an increase in tricarboxylic acid (TCA) cycle activity and higher malate accumulation. On the other hand, the *BraA.cax1a-7* genotype registered a negative response because it showed lower concentrations of AsA and organic acids (OAs) and lower TCA activity. Besides, the higher levels of gibberellin 4 (GA4), and the lower presence of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in *BraA.cax1a-12* plants could also contribute to their higher tolerance to Cd.

The second stress analyzed in this Doctoral Thesis was **salinity stress**. The results showed that different *BraA.cax1a* mutations affected the response to salinity. Thus, the *BraA.cax1a-4* mutation provided better tolerance to the presence of NaCl in the culture medium. This tolerance could be induced by higher Ca and K contents in the shoot, a more efficient ROS detoxification through the superoxide dismutase (SOD) and catalase (CAT) enzymes, a better AsA redox state, and a higher glucose-6-phosphate dehydrogenase (G6PDH) activity. In addition, the *BraA.cax1a-4* mutation provided improved photosynthetic performance manifested by higher values of water use efficiency (WUE), variable fluorescence to maximum fluorescence ratio (Fv/Fm), electron fluxes, and increased accumulation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In contrast, the *BraA.cax1a-7* mutation produced some negative effects on photosynthetic performance, such as higher fluorescence emission values and lower accumulations of Rubisco and soluble carbohydrates, and lower G6PDH activity. Considering the phytohormone profile, *BraA.cax1a-4* plants showed a higher phytohormones concentration that could be related to its better salinity tolerance. Specifically, AIA and gibberellins (GAs) could promote growth

under saline conditions and abscisic acid (ABA) could lead to better stress response and water use efficiency.

As a general **conclusion**, this Thesis described the physiological changes in *B. rapa* produced by different mutations in the CAX1 transporter and its response to different stresses. The *BraA.cax1a-12* mutation was identified as useful for Ca, Mg, and Fe biofortification and Cd phytoremediation, and the *BraA.cax1a-4* mutation was identified to provide enhanced salinity tolerance. In addition, key metabolic processes in the tolerance to each type of studied stress were identified.

INTRODUCCIÓN



1. El TILLING en la mejora vegetal

El incremento de la población mundial es uno de los principales problemas a los que se enfrentará la agricultura en los próximos años. Se estima que para el año 2050 la agricultura deberá aumentar su producción en un 70% para poder alimentar a unos 9.000 millones de habitantes (Hunter *et al.*, 2017). Este problema tiene la dificultad añadida del escenario actual de cambio climático en el que se producirán de forma cada vez más frecuente sequías, inundaciones y variaciones térmicas que pueden provocar grandes pérdidas en la agricultura (Gomez-Zavaglia *et al.*, 2020). Por otro lado, no solo es necesario proveer alimentos en cantidad suficiente, también es importante que estos alimentos sean seguros, nutritivos y de calidad ya que los consumidores están cada vez más informados y más concienciados por su salud y demandan cada vez más alimentos ricos en compuestos bioactivos y/o elementos esenciales y que tengan el potencial de prevenir ciertas enfermedades (Francisco *et al.*, 2017). La biotecnología se postula coma una herramienta esencial para superar estos desafíos. La mejora de las cosechas mediante el desarrollo de variedades más productivas, adaptadas a condiciones adversas como sequía, salinidad, o cultivos que requieran menor cantidad de insumos y que aumenten la acumulación de elementos minerales esenciales permitirán aumentar los rendimientos y la calidad de los productos agrícolas y disminuir el impacto sobre el medio ambiente (Wakeel *et al.*, 2018; Anwar and Kim, 2020). Dentro de la biotecnología, las nuevas técnicas de breeding (NBT) podrían ser herramientas imprescindibles en la mejora vegetal necesaria para superar dichos desafíos.

Una de las técnicas más importantes de las NBTs es el TILLING (Targeting Induced Local Lesions in Genomes) (Holme *et al.*, 2019). Esta técnica se debería potenciar y desarrollar en nuestro país según el Informe de Prospectiva Tecnológica: Impacto de la Biotecnología en los Sectores Agrícola y Ganadero 2025 (Ruiz Galán and Rodríguez, 2013). El TILLING es una herramienta de alto rendimiento y bajo coste de genética inversa en la que se identifica una variación en una secuencia concreta y se trata de asociar a la misma una función biológica o fenotipo. Así, presenta la capacidad de identificar mutantes sin conocimiento previo del fenotipo o el grado de severidad fenotípica y no requiere la obtención de transgénicos (Till *et al.*, 2018). Esto es muy importante en la Unión Europea, ya que existe una estricta normativa en materia de organismos modificados genéticamente (OMG), además de que su percepción social para la producción de alimentos es negativa y tiene un cierto rechazo. El Tribunal de Justicia de la Union Europea en su decisión de 2018 dictaminó que los organismos obtenidos mediante las (NBT), entre las que se incluye el TILLING, no están exentas de la actual Legislación de la UE sobre OGM ya que equiparan sus riesgos y su magnitud a los producidos por los OMGs (Court-of-Justice-of-the-European-Union, 2018). Sin embargo, las NBTs podrían seguir utilizándose con éxito en los proyectos de investigación que se ocupan de la genética molecular de los rasgos de los cultivos. De este modo, podrían usarse para detectar rápidamente genes y rasgos de interés que posteriormente podrían seleccionarse con técnicas de breeding convencional (Holme *et al.*, 2019).

La técnica de TILLING se desarrolla en varias fases. El primer paso es la generación de una población de mutantes (Figura 1A). La generación inicial (M0)

se trata con un agente mutagénico para obtener mutaciones al azar en el genoma de la planta (Till *et al.*, 2018). Se suele utilizar el mutágeno químico etilmetanosulfonato (EMS) que produce mutaciones aleatorias en el material genético por sustitución de nucleótidos; principalmente por alquilación sobre la posición O^6 de la guanina que produce cambios de GC a AT (Stephenson *et al.*, 2010). El objetivo es obtener una alta densidad de mutaciones puntuales inducidas manteniendo una adecuada capacidad de supervivencia y fecundidad. Las semillas mutantes obtenidas mediante esta técnica se germinan (generación M1) y cada planta obtenida se autofecunda para obtener la generación M2 con individuos homocigotos para la mutación seleccionada. Tras su autofecundación se produce una generación M3 sobre la que se realizan los estudios fenotípicos, genéticos y fisiológicos. Durante todo el proceso, se realiza un proceso de "screening" (cribado) masivo de mutaciones de los individuos generados para identificar mutaciones puntuales en genes diana seleccionados (Figura 1B). Debido a la alta densidad de mutaciones de fondo inducidas por el mutágeno, puede ser necesario hacer uno o más retrocruzamientos para correlacionar inequívocamente el genotipo con el fenotipo (Till *et al.*, 2018). El screening se puede llevar a cabo por el método de detección clásico mediante fluorescencia (Till *et al.*, 2003), aunque existen métodos actuales de secuenciación que han permitido rendimientos mucho mayores y la posibilidad de indexar todas las mutaciones en una población TILLING en poco tiempo en lugar de tomar un enfoque de gen a gen. El último paso en el proceso del TILLING es comprobar el efecto de las mutaciones descubiertas en la planta mutante (Figura 1C). Este paso es, y probablemente seguirá siendo, el cuello de botella para el TILLING y es el paso que se va a tratar en la presente tesis (Till *et al.*, 2018).

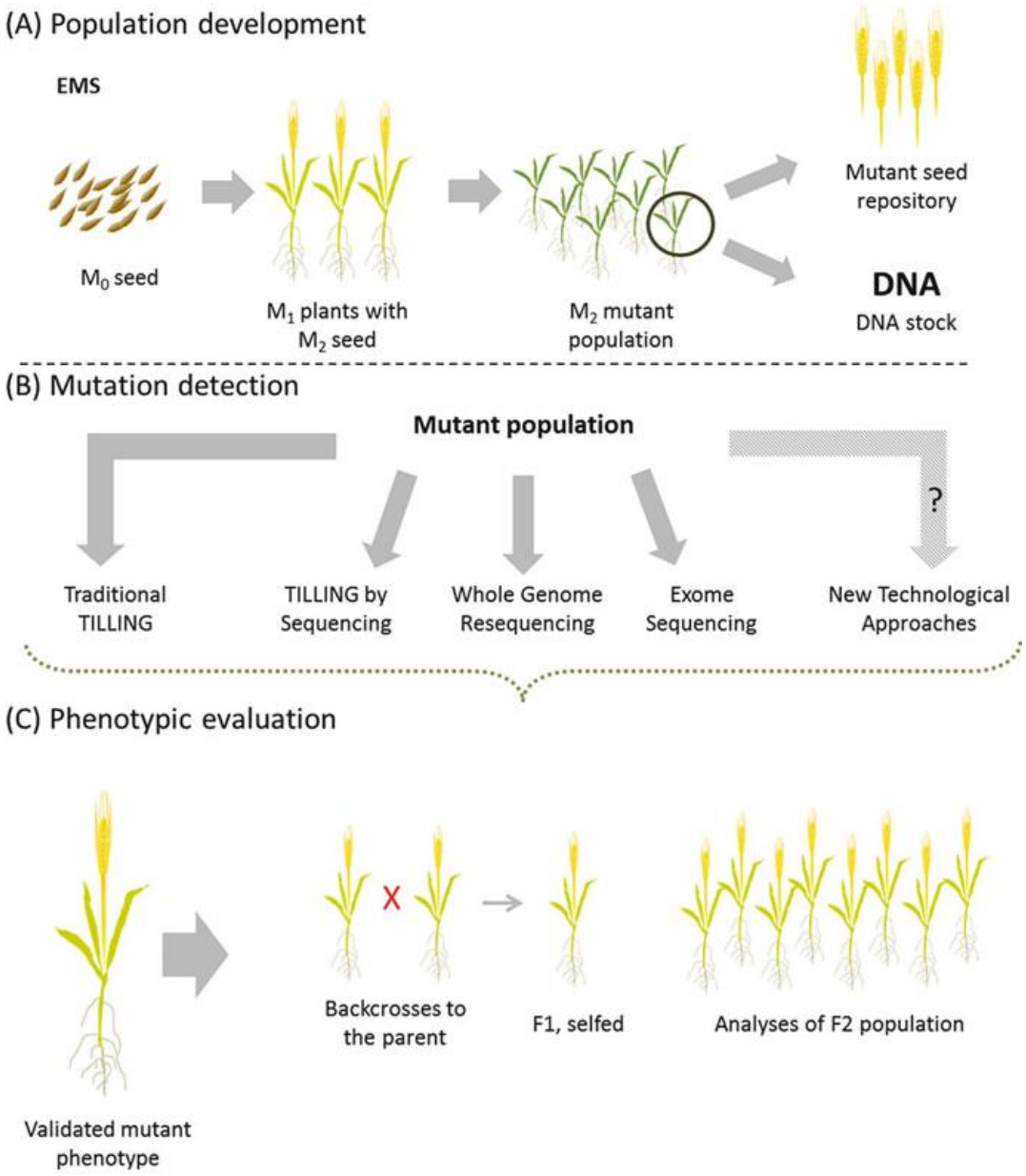


Figura 1. Fases del proceso de la técnica biotecnológica de TILLING. Generación de una población de mutantes (A). Identificación de mutaciones (B). Evaluación fenotípica (C). Adaptado de Till et al. (2018).

2. Papel del Ca en la planta y transportadores vacuolares de Ca (CAX)

El calcio (Ca) es un macronutriente esencial tanto para las plantas como para los animales y que por tanto debe ser obtenido del medio e incorporado en sus organismos. En las plantas, el Ca está presente en las membranas y las paredes celulares desempeñando un papel estructural, y en el citosol siendo crucial en los procesos de señalización celular. La concentración citosólica de Ca debe mantenerse a niveles submicromolares en la célula en reposo a fin de permitir aumentos rápidos para la señalización celular (White and Broadley, 2003). Además, los flujos de Ca también son necesarios en las células guarda para el cierre de los estomas (Dayod *et al.*, 2010). Así pues, debido a la importancia del Ca tanto dentro de la planta, uno de los objetivos de las NBT para la mejora vegetal podría ser la generación de variedades con una homeostasis más eficiente de este elemento. Este objetivo se podría alcanzar mediante la modificación de uno de los principales transportadores de Ca de las plantas, los CAX (CAtion eXchangers) (Pittman and Hirschi, 2016).

Los CAX son miembros de una gran familia de transportadores de cationes y protones. Estos transportadores exportan cationes divalentes, principalmente Ca^{2+}, hacia el interior de la vacuola intercambiándolos por H^{+}, siendo determinantes en la homeostasis celular del Ca^{2+} (Figura 2). También son claves en la eliminación del Ca^{2+} del citosol para generar diferentes concentraciones de Ca^{2+} que sirvan para responder a las señales ambientales o en los procesos de señalización celulares (Conn *et al.*, 2011). Además, los CAX participan en varios

aspectos importantes del crecimiento y desarrollo de las plantas desempeñando un papel clave en la conductancia estomática y en la regulación del pH (Pittman and Hirschi, 2016). Los transportadores CAX obtienen su energía gracias al establecimiento de un gradiente de pH (ácido en el interior) que es generado por bombas de protones en el tonoplasto como la V-ATPasa o la H^+-pirofosfatasa (Figura 2). La actividad de los CAX se ha medido en vesículas de tonoplasto de diferentes especies alejadas filogenéticamente, por lo que se estima su amplia distribución en el reino vegetal. Así pues, la primera isoforma definida de estos transportadores fue CAX1, localizada en vacuolas de *Arabidopsis thaliana* y es la que la posee una mayor actividad de antiporte H^+/Ca^{2+} vacuolar (Manohar *et al.*, 2011). En cuanto a su localización dentro de la planta, este transportador se ha detectado en toda la planta aunque suele ser mayor en hojas, existiendo además una fuerte correlación entre la concentración de Ca y la expresión de CAX1 en los distintos componentes de la hoja (Conn *et al.*, 2011).

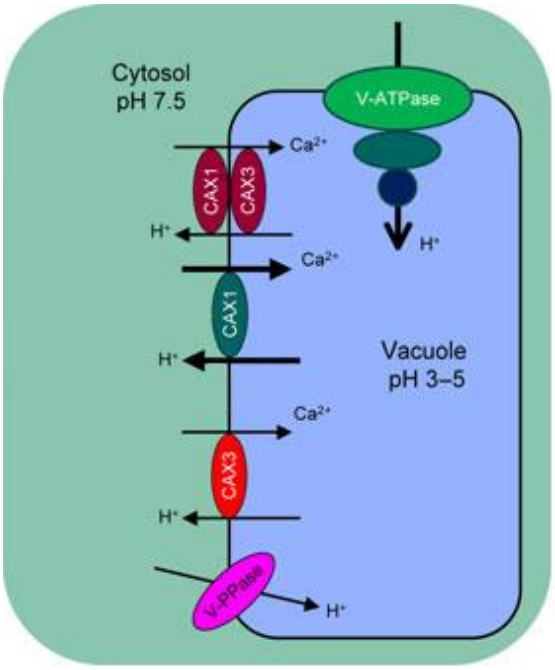


Figura 2. Esquema general del mecanismo de acción de los transportadores CAX en la célula vegetal. Adaptado de Manohar *et al*. (2011).

La estructura de los transportadores CAX está constituida por 11 dominios putativos que atraviesan el tonoplasto y que forman el canal de transporte. En su estructura contienen una región llamada motivo acídico que divide al polipéptido en dos mitades con cierta homología entre sí. Además, en su extremo N-terminal posee un dominio autoinhibitorio hidrofílico que es el principal responsable de la regulación de su actividad. En concreto, se ha observado que la actividad de CAX1 se induce fundamentalmente con el aumento de la concentración de Ca^{2+} citosólica. Esta activación se puede producir mediante la quinasa SOS2 que fosforila el dominio autoinhibitorio activándolo o bien mediante la unión de proteínas CXIP (CAX interaction proteins) (Manohar *et al.*, 2011) (Figura 3). Se ha comprobado que además del Ca^{2+}, un aporte de nitrato, Na^{+}, Ni^{2+} o estrés osmótico en el medio de cultivo favorece la expresión de CAX1 (Catalá *et al.*, 2003).

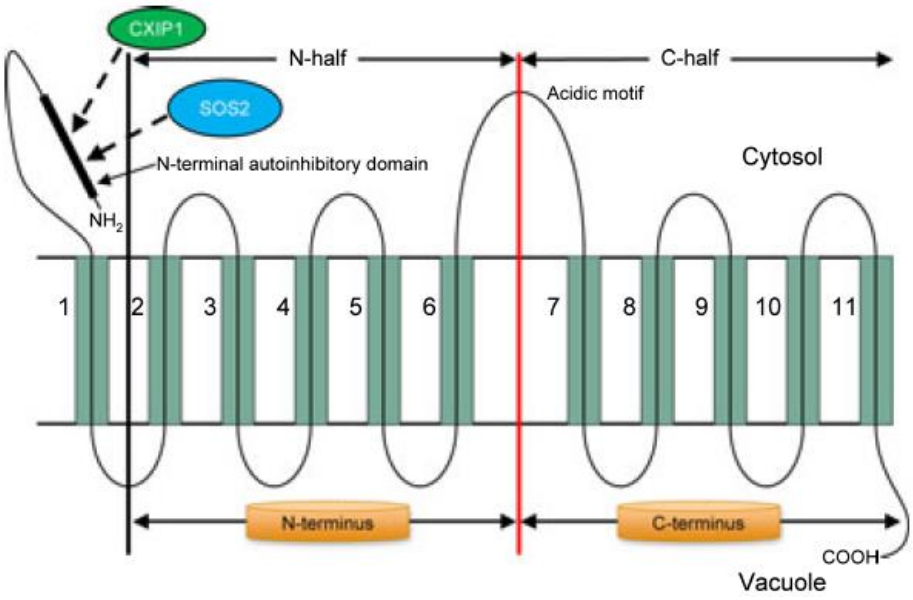


Figura 3. Estructura y regulación de los transportadores CAX (Manohar *et al*., 2011).

Distintos estudios con mutantes knockout para *cax1* han servido para poner de manifiesto la importancia de este transportador. Estos mutantes presentan una menor actividad V-ATPasa porque los H^+ se acumulan más en la vacuola (Cheng *et al.*, 2003). Además, se ha observado que la actividad de los CAX afecta a la composición de la pared celular. Así, en dobles mutantes *cax1cax3* los altos niveles de Ca^{2+} citosólicos producen células más pequeñas y menos densas, probablemente debido a que sus paredes celulares son más gruesas y rígidas por una expresión distinta de proteínas que modifican la pared. Igualmente, la expresión y actividades de los distintos CAX está relacionada entre sí ya que en mutantes knockout *cax1* la falta de actividad de este transportador es compensada en parte con una mayor actividad de otros CAX (Conn *et al.*, 2011) (Figura 4).

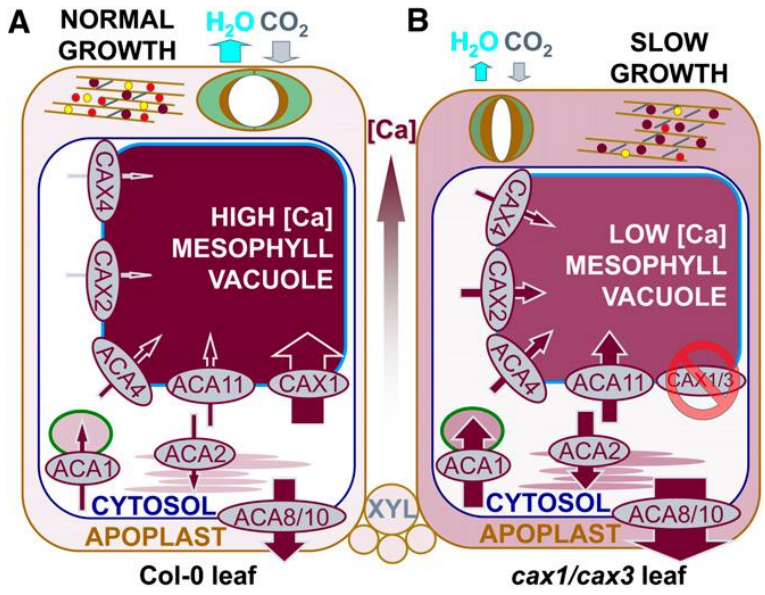


Figura 4. Comparativa entre una célula de una hoja de *A. thaliana* Col-0 (A) y otra célula sin actividad *cax1/cax3* (B). Se representan los efectos de las mutaciones en cax1 y cax3 sobre la actividad de otros transportadores de Ca^{2+}, los flujos de Ca^{2+}, el cierre estomático y los componentes de la pared celular. El tamaño de las flechas indica el flujo de Ca^{2+} a través del transportador (Conn *et al.*, 2011).

Por otro lado, se han realizado una serie de experimentos con plantas de tabaco que expresan una versión de CAX1 de *Arabidopsis* que no tiene el domino autoinhibitorio, por lo que está continuamente activo. En estas plantas se observa una actividad más alta de antiporte Ca^{2+}/H^{+} y asociada a ella una elevada actividad H^{+}/ATPasa vacuolar para suministrar protones. Además, en estos mutantes se producen síntomas de deficiencia de Ca, pero sorprendentemente tienen una mayor concentración de este macronutriente. Este hecho se produce porque el Ca está en gran parte retenido en las vacuolas, bajando los niveles de este elemento en el citosol y por tanto causando su deficiencia fisiológica. Esto hace que las plantas sean más sensibles a los estreses abióticos porque no se pueden producir picos de Ca^{2+} citosólicos y tienen signos de un mayor estrés oxidativo (Hirschi, 1999, 2001; Mei *et al.*, 2007).

La actividad de los transportares CAX tiene un efecto directo en el pH tanto citosólico como apoplástico por lo que influye de manera directa en el transporte y señalización de numerosas fitohormonas como el ácido indolacético (AIA). Así pues, los transportadores CAX aumentan la concentración de H^{+} en el citosol que posteriormente son expulsados al apoplasto por ATPasas de tipo P, produciendo su acidificación. En mutantes knockout *cax1* y *cax3* se ve reducido este transporte de H^{+} y el apoplasto se basifica, lo que causa un defecto en la importación de AIA. El importador de auxinas AUX1 tiene un pH óptimo de funcionamiento entre 5.5-6 y se inhibe a pH mayor. Además, pH 5.5-6 el AIA está protonado lo que permite su entrada en la célula, pero a pH superior predomina la forma no protonada que no puede entrar en la célula y se inhibe el transportador AUX1. En las plantas las auxinas son transportadas principalmente

desde el ápice hacia la base en lo que se conoce como transporte polar. Por lo tanto, en plantas sin actividad CAX1 este transporte polar se ve dificultado presentando menor sensibilidad a las auxinas y debido a que estas producen la inhibición de la elongación del hipocótilo, en las plantas mutantes este se elonga más (Cho *et al.*, 2012). Al mismo tiempo, este efecto sobre las auxinas en los mutantes *cax1* produce un inferior desarrollo de las raíces y un retraso en la floración (Cheng *et al.*, 2003). Por otro lado, los *cax1/cax3* son más sensibles al ácido abscísico (ABA) ya que en ellos el AIA no puede contrarrestarlo. De esta forma, un efecto indirecto en los mutantes *cax1/cax3* es que se promueve el cierre estomático inducido por ABA (Cho *et al.*, 2012). Además, para que se produzca la apertura estomática tiene que entrar K^+ en la célula guarda a consecuencia de una bajada de pH del apoplasto que produce una hiperpolarización de la membrana. Este proceso se ve dificultado en los mutantes *cax1/cax3* que tienen inhibidos los canales de entrada de K^+ y las ATPasas por lo que se promueve el cierre estomático (Conn *et al.*, 2011).

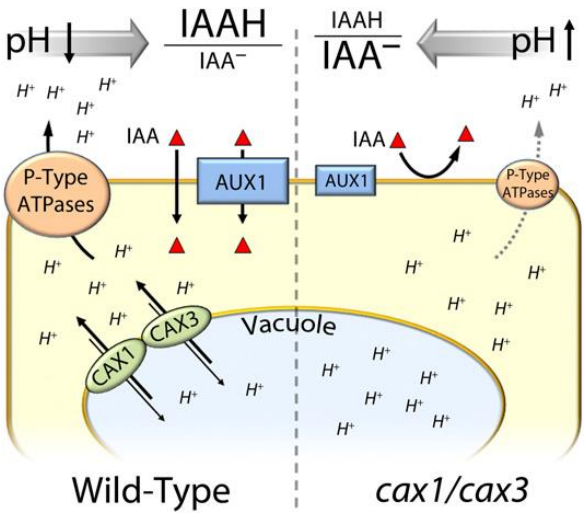


Figura 5. Comparativa entre una célula de una hoja de *A. thaliana* WT (a la izquierda) y otra célula sin actividad *cax1/cax3* (a la derecha). Se representan los efectos de las mutaciones en *cax1* y *cax3* sobre el pH apoplástico y sobre el transporte de AIA. Adaptado de Cho *et al.* (2012).

La gran mayoría de estudios sobre los transportadores CAX se han realizado en plantas de *A. thaliana*, aunque existen algunos estudios en otras especies como en la halófita *Suaeda salsa*. El transportador CAX1 de esta especie vegetal se activa especialmente por salinidad ya que es clave para disminuir el Ca^{2+} citosólico en la respuesta ante estrés (Han *et al.*, 2011). Por otro lado, en *B. oleracea* la expresión de su transportador CAX1 tiene una alta correlación positiva con la concentración de Ca en hojas ya que en la base hay más Ca y más expresión de CAX1. El gen de CAX1 de esta especie tiene muchos más elementos reguladores en cis que el de *A. thaliana*. Además, CAX1 tiene una actividad mayor en la variedades más resistentes a la deficiencia de Ca (Lee *et al.*, 2013). En cuanto a la especie utilizada en la presente tesis (*Brassica* rapa), se identificó a *cax1* como un loci de rasgo cuantitativo de expresión que se ve afectado por la concentración externa de Ca (Graham *et al.*, 2014).

3. Influencia de CAX y del Ca en el metabolismo de la planta

Debido a la importancia fisiológica del Ca y del transportador CAX1 su modificación puede afectar a los distintos procesos metabólicos de la planta entre los que destacan: la fotosíntesis, los procesos de estrés oxidativo, el metabolismo del nitrógeno (N), el ciclo de los ácidos tricarboxilicos (TCA) y la regulación fitohormonal.

3.1. Fotosíntesis

La fotosíntesis es uno de los procesos más importantes en la planta mediante el cual se producen compuestos orgánicos y energía química aprovechable, usando como fuente de energía la luz del sol. Se divide en dos partes o etapas: por un lado, la etapa fotoquímica en la que las moléculas de clorofila captan la luz y la transfieren a través de la cadena de transporte electrones para producir energía química en forma de NADPH y ATP. La segunda etapa es la fase de fijación fotosintética del CO_2 en la que este gas es asimilado en el ciclo de Calvin-Benson, en el que interviene la ribulosa-1,5-bifosfato carboxilasa/oxigenasa (Rubisco) y en la que se forman los primeros asimilados del carbono (Stirbet *et al.*, 2020). Para estudiar la etapa fotoquímica, se recurre al análisis de la fluorescencia de la clorofila *a* que refleja el estado de la fotosíntesis y los efectos en ella debidos al estrés. Cuando se perturba el metabolismo de las plantas, la fluorescencia disipa el exceso de energía no asimilable para evitar daños. Se puede estudiar la curva de fluorescencia emitida que tiene varias fases (OJIP) y de la que se derivan diversos parámetros que indican el rendimiento in vivo del fotosistema II (PS II) (Strasser *et al*., 2004).

El Ca tiene una gran influencia en la fotosíntesis. Así, el Ca forma parte del cluster de Mn_4-Ca en el complejo productor de oxigeno (OEC) del PS II (Ferreira *et al*., 2004), por lo que una deficiencia de Ca podría afectar a la función del OEC y por tanto a la fotolisis del agua. Este fenómeno se observa como un pico característico en la curva de fluorescencia a los 300 µS (pico K). Por otro lado, Tan *et al*. (2011) observaron que la aplicación de Ca exógeno puede proteger la

fotosíntesis contra el estrés mejorando el transporte electrónico. El Ca ayuda a reparar el complejo PS II, a mantener la fotosíntesis y a activar las enzimas antioxidantes. Esto último es importante ya que las especies reactivas de oxígeno (ROS) causan daños en los fotosistemas e inhiben su reparación (Gururani *et al*., 2015).

El Ca también es necesario para un correcto flujo lineal de electrones fotosintéticos (LEF) a través de los fotosistemas. Asimismo, este elemento es necesario para el flujo cíclico de electrones fotosintéticos (CEF) que también produce ATP y es importante para evitar la reducción excesiva del PSI bajo condiciones ambientales estresantes. (Terashima *et al.*, 2012). Durante la fotosíntesis, el gradiente de pH que se produce no sólo se utiliza para la producción de ATP, sino también para la importación de Ca^{2+} hacia el lumen. El Ca^{2+} se une a la membrana tilacoidal y a los canales para el bombeo de protones activándolos y varios componentes de la cadena de transporte son fosforilados de manera dependiente de Ca. Estas proteínas fosforiladas interactúan con las proteínas de la antena para modular la energía de excitación hacia el centro de reacción (Figura 6A) (Hochmal *et al.*, 2015).

Por otro lado, la etapa de fijación fotosintética del CO_2 se puede analizar midiendo el flujo de CO_2 de las hojas. Este análisis se realiza mediante un analizador de gases por infrarrojos (IRGA). La reducción en el flujo de CO_2 entrante en la hoja suele indicar una menor tasa fotosintética (Stirbet *et al.*, 2020). En esta etapa el Ca^{2+} también desempeña un papel clave ya que se

necesita una concentración de Ca^{2+} libre baja (~150 nM) en el estroma para la activación de la fijación de carbono fotosintético por las enzimas del ciclo de Calvin-Benson. Cuando hay luz, el Ca^{2+} es introducido desde el citosol al cloroplasto y almacenado en el lumen de los tilacoides o en proteínas de unión al Ca^{2+}. Este aumento en el Ca^{2+} cloroplástico probablemente no está afectando a la concentración de Ca^{2+} libre del estroma, porque el Ca^{2+} es rápidamente transportado hacia el lumen del tilacoide y/o secuestrada por las proteínas de unión de Ca^{2+} como las proteínas sensoras de Ca^{2+} (CAS) (Figura 6B). Las CAS han sido descritas como sensores extracelulares de Ca^{2+} por lo que son cruciales para la regulación estomática adecuada en respuesta a las elevaciones de Ca^{2+} externo mediante la modulación de los flujos citoplasmáticos del Ca^{2+}. En oscuridad, el Ca^{2+} es liberado incrementando su concentración en el estroma a 2-6 µM lo que tiene un efecto inhibitorio en las enzimas del ciclo de Calvin-Benson. Posteriormente el Ca^{2+} del estroma pasa al citosol para restaurar sus niveles normales (Figura 6C). Además, se cree que el Ca^{2+} es importante para la regulación de varias enzimas clave, entre ellas la fructosa 1,6-bisfosfatasa (Hochmal *et al.*, 2015).

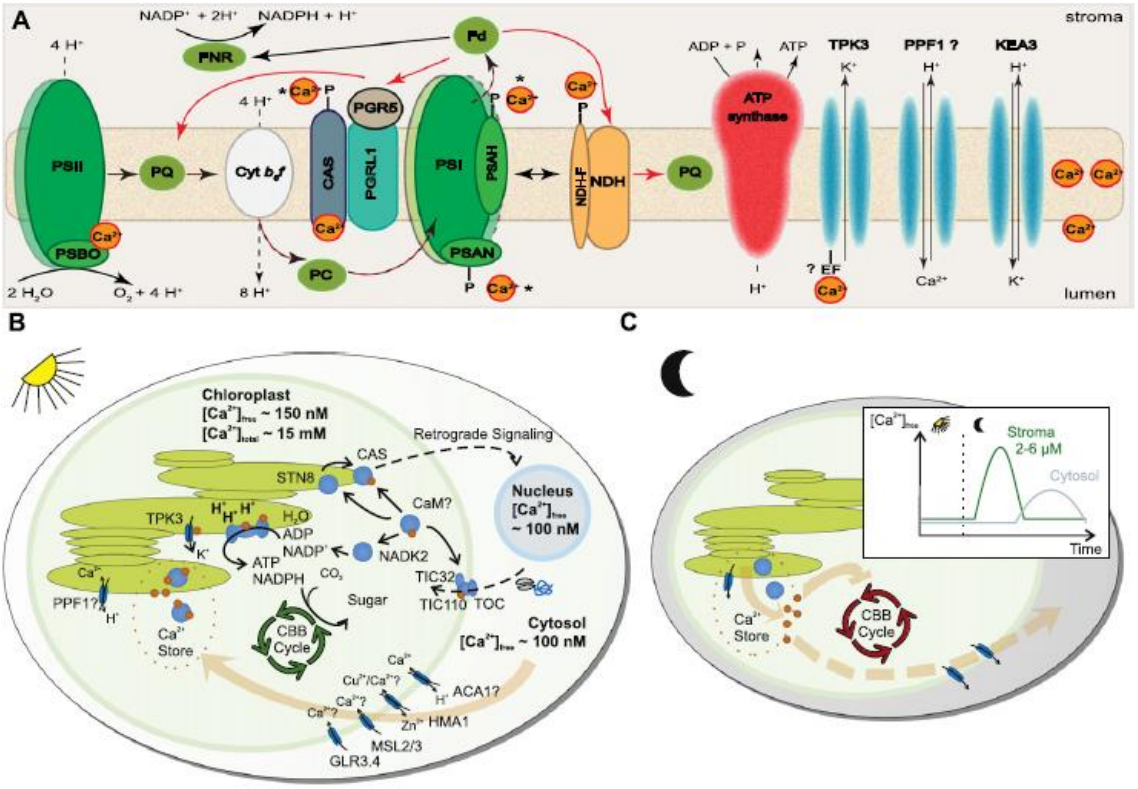


Figura 6. Representación de los procesos fotosintéticos regulados por Ca^{2+} en la planta. Papel del Ca^{2+} en la cadena de transporte de electrones (A). Las flechas negras representan el LEF y las rojas el CEF. Dinámica del Ca^{2+} en el cloroplasto durante el día (B) y durante la noche (C). Adaptado de Hochmal *et al.* (2015).

3.2. Estrés oxidativo

Durante los procesos de estrés, los electrones que tienen un estado de alta energía se transfieren al oxígeno molecular (O_2) para formar ROS. Los ROS como el oxígeno singlete ($^1O_2$), el ión superóxido ($O_2^{\cdot-}$) y los peróxidos como el peróxido de hidrogeno (H_2O_2) son moléculas tóxicas para la planta. Los ROS atacan a compuestos como los lípidos de membrana o el ADN, con la formación de peróxidos de lípidos o nucleótidos (Chakradhar *et al.*, 2017). Además, los ROS inhiben la biosíntesis de las clorofilas y a los fotosistemas, disminuyendo el rendimiento fotosintético (D'Alessandro *et al.*, 2013). Por tanto, la capacidad que tienen las plantas de contrarrestar el estrés oxidativo evitando que se generen

ROS o eliminándolos es básica para el funcionamiento normal de las plantas. Así pues, las plantas poseen dos tipos de mecanismos para eliminar los ROS. Por un lado, la acumulación de compuestos con actividad antioxidante como el glutatión (GSH) y el ascorbato (AsA) entre otros y por otro, la síntesis y activación de enzimas antioxidantes como la superóxido dismutasa (SOD) que eliminan $O_2^{\bullet -}$ y la catalasa (CAT) y las peroxidasas que eliminan el H_2O_2 (Chakradhar *et al.*, 2017). Gran parte de estos sistemas antioxidantes participan en el ciclo del AsA/GSH también conocido como ciclo de Foyer-Halliwell-Asada (Figura 7). El ciclo AsA/GSH es una de las principales formas de reciclar los antioxidantes en las plantas y de eliminar los ROS manteniendo el estado reducido en las células que se suele perder durante el estrés oxidativo. Las enzimas del ciclo AsA/GSH como la glutatión reductasa (GR) son capaces de regenerar antioxidantes no enzimáticos. Además, el GSH reduce los peróxidos y participa en la activación de los genes relacionados con la respuesta al estrés y el AsA elimina los ROS directamente e indirectamente a través del ciclo AsA/GSH (Gill and Tujeta, 2010; Sharma *et al.*, 2012).

Un estudio realizado por Pokotylo *et al*. (2012) demostró que una expresión alterada de *CAX1* puede conducir a un mayor estrés oxidativo, como se observó en los mutantes de tabaco *cax1* que presentaban mayores niveles de malondialdehido (MDA) y menores actividades de enzimas antioxidantes. El Ca^{2+} está relacionado con la concentración de ROS, ya que ambos están implicados en la señalización celular del estrés (Gilroy *et al.*, 2014). Así, existen diversas quinasas dependientes de Ca^{2+} (CDPKs) que regulan la producción de ROS. Algunas pueden promover su acumulación, por ejemplo, en procesos de

señalización o muerte celular y otras pueden reducir esta acumulación ante procesos de tolerancia al estrés (Atif *et al.*, 2019). Por otra parte, algunos estudios mostraron que el Ca aplicado en dosis adecuadas puede ser beneficioso para reducir el estrés oxidativo y evitar la acumulación de ROS (Tan *et al.*, 2011; Srivastava *et al.*, 2015). Igualmente, se ha observado que los niveles de GSH y la relación GSH/GSSG se ven afectados por los niveles de Ca, ya que se reducen en caso de deficiencia de este elemento (Cho *et al.*, 2012).

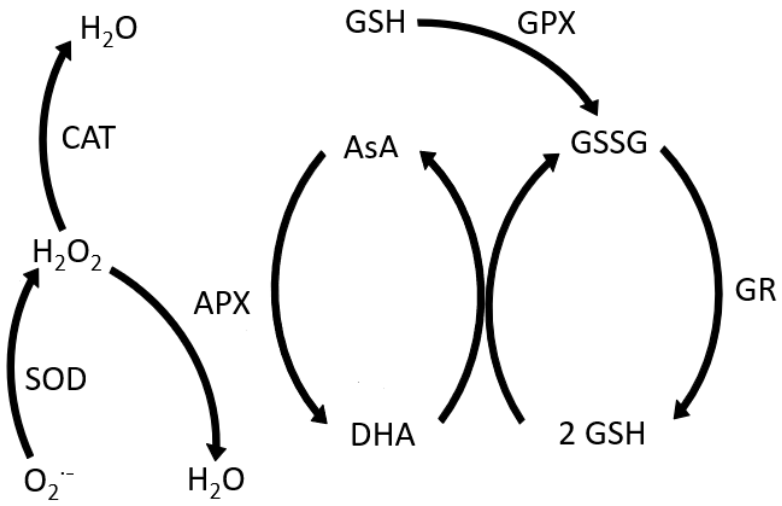


Figura 7. Esquema simplificado que representa el ciclo de Foyer-Halliwell-Asada y la detoxificación de ROS. Superóxido dismutasa (SOD), Catalasa (CAT), Ascorbato peroxidasa (APX), ascorbato reducido (AsA), ascorbato oxidado (DHA), Glutation reducido (GSH), Glutation oxidado (GSSG), Glutation reductasa (GR), Glutation peroxidasa (GPX).

3.3. Metabolismo del Nitrógeno

Uno de los procesos más importantes dentro del metabolismo de la planta que es afectado por la homeostasis del Ca es el metabolismo del N (Gao *et al.*, 2011; Krouk, 2017). Una correcta regulación del metabolismo del N es básica

dado que es un determinante importante de la productividad de los cultivos y el N es la base de componentes tan importantes como los aminoácidos (AAs), las proteínas y los ácidos nucleicos. Así, los principales asimilados del N son los AAs como el glutamato (Glu), la glutamina y el ácido aspártico (Asp) (Figura 8) (Beatty and Wong, 2017). Varias investigaciones demostraron que tanto la disponibilidad de Ca^{2+} como su homeostasis pueden afectar al metabolismo del N. De esta manera, la actividad nitrato reductasa (NR) depende de CDPKs que fosforilan a esta enzima desactivándola en oscuridad (Sanders *et al.*, 2002). Además las enzimas de asimilación del amonio, glutamato deshidrogenasa (GDH) y glutamina sintetasa (GS), también son activadas por Ca^{2+} (Zhang *et al.*, 2013). Así pues, las aplicaciones deficientes o tóxicas de Ca^{2+} causan la inhibición de las enzimas del metabolismo del N (Gao *et al.*, 2011; Chao *et al.*, 2008). En este sentido, Gao *et al.* (2011) observaron en plantas de melón que la deficiencia de Ca reducía la disponibilidad de AAs libres. Por otro lado, la homeostasis del Ca^{2+} es tan importante como sus niveles, por lo que la aplicación de compuestos como los oligosacáridos de alginato activan el metabolismo del N y aumentan la concentración total de N al mejorar el flujo de Ca^{2+} hacia el citosol (Zhang *et al.*, 2011). Además, en estrecha relación con el metabolismo del N, la fotorrespiración es importante para mantener una homeostasis adecuada del N y producir metabolitos para otros procesos. Así, en la fotorrespiración se produce NH_4^+ que es asimilado en el cloroplasto por las enzimas del ciclo GS/GOGAT (Figura 8).

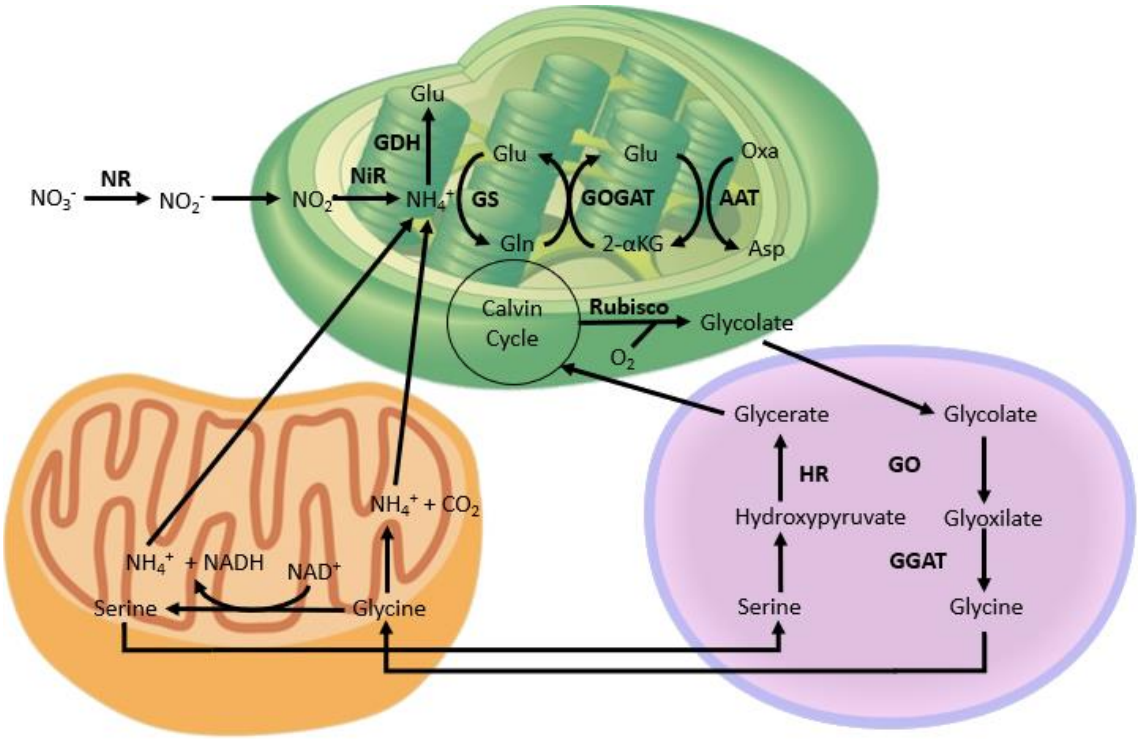


Figura 8. Representación esquemática del metabolismo del N y de la fotorrespiración en plantas. Nitrato reductasa (NR), nitrito reductasa (NiR), glutamato deshidrogenasa (GDH), glutamina sintetasa (GS), glutamato sintasa (GOGAT), aspartato aminotransferasa (AAT), glioxilato oxidasa (GO), glutamato: glioxilato aminotransferasa (GGAT), hidroxipiruvato reductasa (HR).

3.4. Ciclo de los ácidos tricarboxílicos

Muy ligado al metabolismo del N, otro de los procesos regulados por Ca en la planta es el ciclo de Krebs o también conocido como ciclo de los ácidos tricarboxilicos (TCA) (Figura 9). Este proceso se produce en las mitocondrias de las células y desempeña un papel fundamental en la fisiología de las plantas, ya que es crucial en la producción de energía, proporciona precursores de AAs y está estrechamente relacionado con otros procesos importantes como la fotorrespiración y el equilibrio iónico (Igamberdiev and Eprintsev, 2016). El ciclo de los TCA es un proceso altamente regulado, y una de las formas más importantes de regulación es a través de los flujos de Ca^{2+} (Sweetlove *et al.*,

2010). Así, se ha comprobado que el suministro de Ca^{2+} influye en las actividades enzimáticas de este proceso en *B. rapa* (Blasco *et al.*, 2015). La actividad del TCA está determinada por la proporción NADPH/$NADP^+$ y uno de los factores que determinan esta proporción es la actividad NADPH deshidrogenasa que es regulada por Ca^{2+}. Además, a través del Ca^{2+} también se regula la actividad del ciclo como respuesta a un estrés (Bykova and Igamberdiev, 2016). Igualmente, otras deshidrogenasas como la piruvato deshidrogenasa, la NADH-isocitrato deshidrogenasa, y la α-cetoglutarato deshidrogenasa pueden ser activadas por el aumento de concentración de Ca^{2+} en la mitocondria (Hochmal *et al.*, 2015).

Los AAs son utilizados como sustratos para formar compuestos que son catabolizados por las enzimas del ciclo del TCA para producir energía para el crecimiento de las plantas, especialmente en respuesta al estrés. De esta forma, el metabolismo del N se conecta con la respiración a través del ciclo de los TCA (Galili *et al.*, 2016). Asimismo, la enzima fosfoenolpiruvato carboxilasa (PEPC) forma parte de una importante vía anaeróbica por la que se produce oxalacetato (Figura 9). Además, esta enzima puede fijar el CO_2 de la fotorrespiración y actúa como factor de enlace entre el metabolismo del C y el N, ya que el oxalacetato producido es un precursor del malato, el citrato y el Asp (Sweetlove *et al.*, 2010). Así pues, el citrato, el malato y el oxalato son tres ácidos orgánicos (OAs) que son a la vez intermediarios del ciclo de los TCA e intervienen en la tolerancia a la toxicidad de los metales, la solubilización de los nutrientes en el suelo en condiciones de deficiencia, su transporte a través del xilema y su secuestro vacuolar (Evangelou *et al.*, 2007). Por otra parte, la formación de cristales de oxalato tiene una función de almacenamiento de Ca^{2+} en las vacuolas, así como

el mantenimiento del gradiente de concentración entre la vacuola y el citosol (Igamberdiev and Eprintsev, 2016).

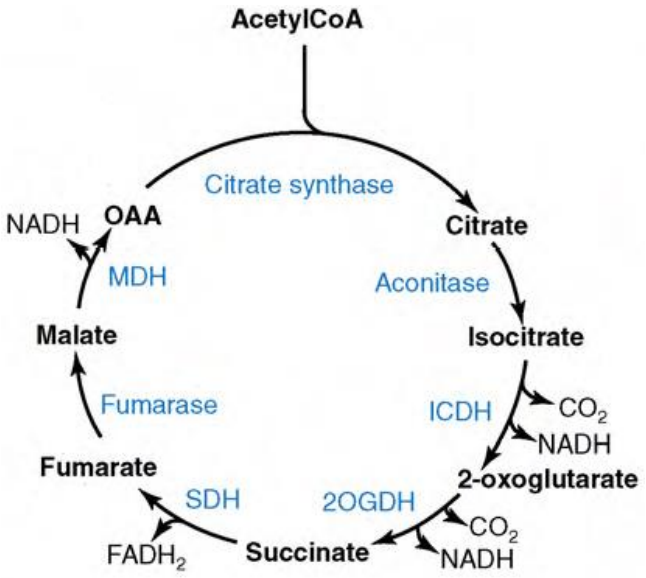


Figura 9. Esquema representativo del ciclo de los ácidos tricarboxílicos. Adaptado de Sweetlove *et al.* (2010).

3.5. Fitohormonas

Además del Ca^{2+}, las fitohormonas son otro de los elementos fundamentales que actúan junto con este elemento en la regulación del metabolismo de las plantas. Las fitohormonas son compuestos de diversa naturaleza que regulan el metabolismo general de las plantas y que se caracterizan por actuar a concentraciones muy bajas. Se han descrito multitud de compuestos que funcionan como fitohormonas, pero los más estudiados pertenecen a cinco grupos: auxinas, giberelinas (GAs), citoquininas (CKs), ABA y etileno (Ku *et al.*, 2018). Estos compuestos intervienen tanto en el control del crecimiento y desarrollo de la planta, como en las respuestas al estrés y la senescencia. Además, las fitohormonas inducen cambios en el metabolismo de

las plantas para mejorar la absorción de nutrientes y la homeostasis dentro de la planta y, a su vez, nutrientes como el Ca^{2+} afectan a la síntesis y la acción de las fitohormonas (Marschner, 2012). Cheng *et al.*, 2003 observaron que la señalización de una auxina como el AIA se ve alterada en mutantes *cax1* de *A. thaliana*. De igual modo, estos mutantes perdieron sensibilidad al etileno y al ABA (Zhao *et al.*, 2008). Por otro lado, el Ca^{2+} es capaz de activar la síntesis hormonal y actúa en cascadas de transducción de señales en respuesta a las fitohormonas. De esta manera, el Ca^{2+} participa en la señalización del ABA, las GAs, el AIA y el etileno a través de CDPKs (Xu and Huang, 2017). Asimismo, las CKs y las GAs, específicamente la forma bioactiva GA3, influyen en la acumulación de Ca^{2+} en algunas especies (Yang *et al.*, 2011). Por otro lado, el ABA induce la elevación de los niveles de Ca^{2+} citosólico, lo que se debe en parte a la liberación de Ca^{2+} de la vacuola, por lo que el ABA influye en la distribución celular del Ca^{2+} (de Freitas *et al.*, 2013).

4. Papel del Ca y de CAX1 en la mejora vegetal

Se ha comprobado que plantas mutantes de *Arabidopsis* en las cuales se han silenciado genes *CAX* como *CAX1* y *CAX3* son muy sensibles a la presencia de diferentes estreses abióticos. Esta mayor sensibilidad probablemente es debida a que no se pueden producir variaciones en los niveles de Ca^{2+} citosólicos dificultando la transducción de señales en la célula (Cheng *et al.*, 2003; Park *et al.*, 2005; Zorrilla *et al.*, 2019; Gao *et al.*, 2019). Por tanto, los transportadores CAX parecen jugar un papel esencial en las respuestas de las plantas ante un estrés (Cheng *et al.*, 2005; Zhao *et al.*, 2008). Asimismo, se ha postulado que la

manipulación de los niveles de expresión de los transportadores CAX puede tener un efecto positivo en la productividad de la planta y/o la calidad nutricional de los alimentos. Por otro lado, estas modificaciones pueden cambiar la especificidad de sustrato y por tanto las plantas resultantes pueden ser útiles para la fitorremediación de suelos contaminados con metales pesados. En concreto, el transportador CAX1 parece tener un intervalo bastante amplio de especificidad para diferentes cationes divalentes incluyendo varios metales pesados, lo que convierte a este transportador en un excelente candidato para tratar su manipulación en programas de fitorremediación de elementos como el cadmio (Cd) (Manohar *et al.*, 2011).

Otro mecanismo por el que CAX1 podría mejorar la resistencia al estrés sería mediante la inducción de la absorción y acumulación de Ca^{2+} (fortificación con Ca^{2+}) (Manohar *et al.*, 2011). Según esta línea de trabajo, la inducción de los transportadores CAX produciría un aumento en la concentración de Ca^{2+} en las plantas lo que reduciría el efecto negativo de estreses abióticos tales como la salinidad y la presencia de metales pesados. En este sentido, existen varios trabajos en los que se muestra que el aumento de Ca favorece las respuestas de tolerancia en plantas. Por ejemplo, ante un estrés por salinidad la aplicación de Ca favorece la acumulación de prolina y de K e induce la actividad antioxidante mejorando la tolerancia al estrés (Acosta-Motos *et al.*, 2017).

Por lo tanto, el transportador CAX1 es un objetivo potencial para obtener plantas con mejor homeostasis del Ca o con mayores concentraciones de este

elemento en las partes comestibles (Pittman y Hirschi, 2016). Mediante el TILLING se pueden modificar los niveles de expresión y la especificidad al sustrato de este transportador, lo que podría tener un efecto positivo en la productividad de la planta, ya sea incrementando la calidad nutricional de los alimentos (biofortificación), mejorando su resistencia a estreses abióticos como la salinidad o eliminando metales tóxicos del suelo como el Cd (fitorremediación) (Manohar *et al.*, 2011).

4.1. Biofortificación

El Ca, así como el hierro (Fe), el magnesio (Mg) y el zinc (Zn) son elementos esenciales para la salud humana. Se ha estimado que dos tercios de la población mundial tienen una dieta deficiente de al menos uno de estos elementos, lo que aumenta el riesgo de padecer ciertas enfermedades (White and Broadley, 2009). Por ello, la biofortificación es una técnica muy útil para aumentar determinados nutrientes en las partes comestibles de los cultivos. Esto puede conseguirse de dos maneras que son complementarias: proporcionando un suministro adecuado de nutrientes y/o elementos beneficiosos, o mediante la mejora de los cultivos, por mejora tradicional o mediante OMG y las NBTs (Sharma and Verma, 2019).

La importancia de la realización de programas de biofortificación con Ca^{2+} en plantas destinadas al consumo humano radica en la esencialidad de este catión en la salud humana. Una de las principales funciones beneficiosas de la ingesta de Ca es la reducción del riesgo de padecer osteoporosis que es una

enfermedad sistémica caracterizada una mayor debilidad ósea y un riesgo más elevado de fracturas (Broadley and White, 2010). Se ha estimado que más de 20 millones de personas sufren osteoporosis en la Unión Europea. En concreto, en España se ha observado que las personas de más edad solo cubren el 75% de la ingesta recomendada de Ca y se espera un aumento del 30% en las fracturas óseas para el año 2030 (Romero-Marco and Pérez-Gallardo, 2020). Uno de los factores de riesgo más importantes que predispone al desarrollo de esta enfermedad es una dieta pobre en Ca, por lo que uno de los consejos principales para detener el avance de la osteoporosis y prevenir las fracturas es tomar alimentos ricos en este elemento mineral.

La mejor opción para el desarrollo de programas de biofortificación con Ca es aumentar su concentración en las vacuolas celulares ya que el transporte hacia este orgánulo disminuye la concentración citosólica de Ca^{2+} lo que activa a transportadores de Ca^{2+} de la membrana plasmática. Hay dos tipos de transportadores vacuolares los ACA y los CAX. Los transportadores CAX son más eficientes porque requieren menos energía para su actividad (Dayod *et al.*, 2010). Además, existe una fuerte correlación entre la expresión de los CAX y la acumulación de Ca. Por ejemplo, las plantas de *B. rapa* tienen una mayor acumulación de Ca en las células en empalizada del mesófilo de las hojas (Rios *et al.*, 2012), donde la expresión de CAX es superior (Conn *et al.*, 2011). Por lo tanto, CAX1 es un objetivo potencial para obtener plantas con mayores concentraciones de Ca en las partes comestibles (Pittman y Hirschi, 2016).

Existen trabajos previos que muestran el potencial de la manipulación del gen CAX1 en el incremento de Ca en la parte comestible y por lo tanto su validez en programas de biofortificación con Ca. Así pues, experimentos con plantas de tomate y de patata a las que se les insertó una versión de CAX1 sin el domino inhibitorio consiguieron aumentar considerablemente la acumulación de Ca en las partes comestibles. Sin embargo, estas plantas presentaban desperfectos en los frutos debido a una deficiencia de Ca^{2+} citosólica (Park *et al.*, 2005; Zorrilla *et al.*, 2019; Gao *et al.*, 2019). Para evitar los efectos negativos que se producen al sobreactivar a CAX1 se han conseguido fenotipos intermedios, modificando AAs concretos del domino regulador de CAX1. En estos experimentos las plantas no acumularon tanto Ca como las que sobreexpresaban CAX1 pero no tuvieron tantos efectos negativos (Mei *et al.*, 2007). Por otro lado, hay experimentos similares a los anteriormente descritos pero en los que no se observan efectos negativos, por ejemplo en patata (Park *et al.*, 2005), en lechuga (Park *et al.*, 2009) y en zanahoria (Morris et al., 2008). Estos dos últimos estudios tuvieron en cuenta otros dos factores importantes además de la acumulación de Ca como son el sabor y la textura y la asimilación posterior del Ca por los animales. De hecho, es muy importante tener en cuenta no solo que las plantas tengan una concentración de Ca más elevada si no que este Ca sea absorbido de manera efectiva en el sistema digestivo al ser consumido. De esta forma, la biofortificación con Ca también se puede conseguir disminuyendo compuestos que impiden su absorción como los fitatos y el oxalato (Sharma *et al.*, 2017). Otro factor a tener en cuenta es que la gran mayoría de estudios se han realizado con plantas transgénicas. Una posible alternativa al uso de los transgénicos sería la generación de mutantes con una modificación de la actividad del CAX1 que

afectara a su autorregulación o actividad que podría ser obtenido con técnicas como el TILLING.

4.2. Toxicidad por cadmio

Los metales pesados son importantes contaminantes ambientales que reducen la productividad de los cultivos y constituyen una amenaza para toda la cadena alimentaria, incluida la salud humana. Uno de los metales pesados más tóxicos es el Cd, siendo uno de los más peligrosos debido a su gran movilidad y disponibilidad para todos los organismos vivos. En las plantas, el Cd produce graves síntomas de toxicidad, como clorosis, reducción del crecimiento y deterioro de los procesos fisiológicos como la fotosíntesis, la respiración, la transpiración y la absorción de nutrientes (Clemens and Ma, 2016). Asimismo, el Cd desencadena la acumulación de ROS produciendo estrés oxidativo (Sandalio *et al.*, 2009). Para evitar el efecto tóxico del Cd, las plantas aumentan la producción de GSH, fitoquelatinas (PC) y OAs como el citrato, el malato y el oxalato. Estos compuestos forman complejos metalorgánicos que mitigan el efecto tóxico de los metales pesados y facilitan su transporte y almacenamiento en vacuolas y, por lo tanto, previenen su efecto tóxico (Mahmud *et al.*, 2018)(Li *et al.*, 2020).

La fitorremediación es una estrategia respetuosa con el medio ambiente para limpiar los suelos contaminados con metales pesados (Azevedo *et al.*, 2012). La planta ideal para fines de fitorremediación debe ser capaz de tolerar el contaminante, acumularlo en una parte fácilmente cosechable, estar adaptada

para crecer localmente y producir una alta biomasa (Thakur *et al.*, 2019). Así, especies como *B. rapa* son adecuadas para la fitorremediación del Cd ya que toleran niveles relativamente altos de este metal y lo acumulan en una concentración de hasta 100 ppm (Baker *et al.*, 2000; D'Alessandro *et al.*, 2013). Los investigadores están generando y seleccionando nuevas variedades con mayor capacidad de fitorremediación mediante la modificación de ciertos genes clave (Ali-Zade *et al.*, 2010). Un objetivo potencial para estas investigaciones son los transportadores CAX (Baliardini *et al.*, 2015).

La modificación del transportador CAX podría tener un gran impacto en la tolerancia al Cd debido a que el Cd y el Ca son elementos antagónicos, ambos elementos tienen características físicas similares y compiten entre sí por su entrada en las plantas (Tran and Popova, 2014). Una concentración elevada de Ca^{2+} puede tener potencialmente un efecto protector sobre las células expuestas a Cd^{2+} al bloquear parcialmente su captación (Tian *et al.*, 2016; Huang *et al.*, 2017). Igualmente, una mayor aplicación de Ca reduce la absorción y concentración de Cd en plantas de *Sedum alfredii*, induce su actividad antioxidante y ayuda a mantener la integridad celular (Tian *et al.*, 2011). Además, el Ca puede aliviar indirectamente los efectos tóxicos del Cd debido a su relación con la producción de ROS (Rodríguez-Serrano *et al.*, 2009) (Figura 10). Así pues, el estudio de Ahmadi *et al.* (2018) demuestra el papel clave del transportador CAX1 en la reducción de la acumulación de ROS ante toxicidad por Cd. Asimismo, plantas mutantes de *Arabidopsis* con una mayor actividad de CAX1 poseen una mejor adaptación a suelos ricos en serpentina lo que sugiere una mayor tolerancia a la presencia de metales pesados (Bradshaw, 2005). Por

último, la razón más importante es que CAX1 es capaz de transportar Cd dentro de la vacuola. De este modo, en dos experimentos independientes, Shigaki *et al.* (2005) y Wu *et al.* (2011) obtuvieron variantes de CAX1 con una capacidad mejorada de transporte de Cd. Sin embargo, las plantas presentaban una mayor tolerancia frente al Cd. Estos autores sugieren que CAX1 podría transportar Cd a la vacuola de las células como mecanismo de protección. De hecho, las plantas de petunia en el estudio de Wu *et al.* (2011) fueron capaces de acumular hasta 500 mg de Cd por Kg de planta por lo que podrían ser útiles para fines de fitorremediación.

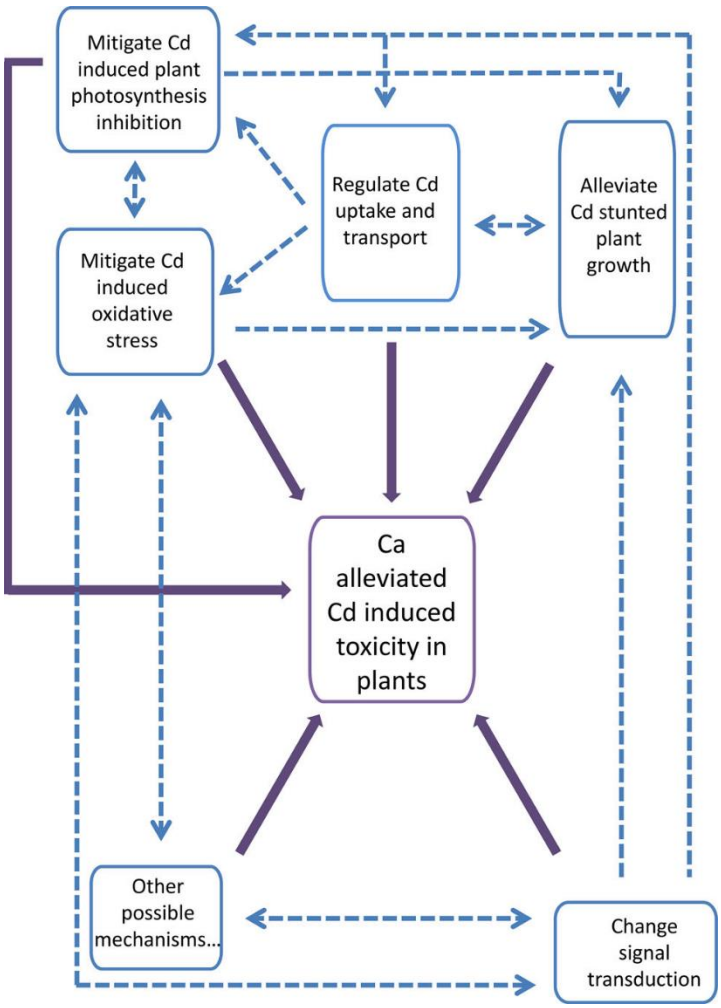


Figura 10. Mecanismos por los cuales el Ca alivia la toxicidad de cadmio en plantas (Huang *et al.*, 2017).

4.3. Salinidad

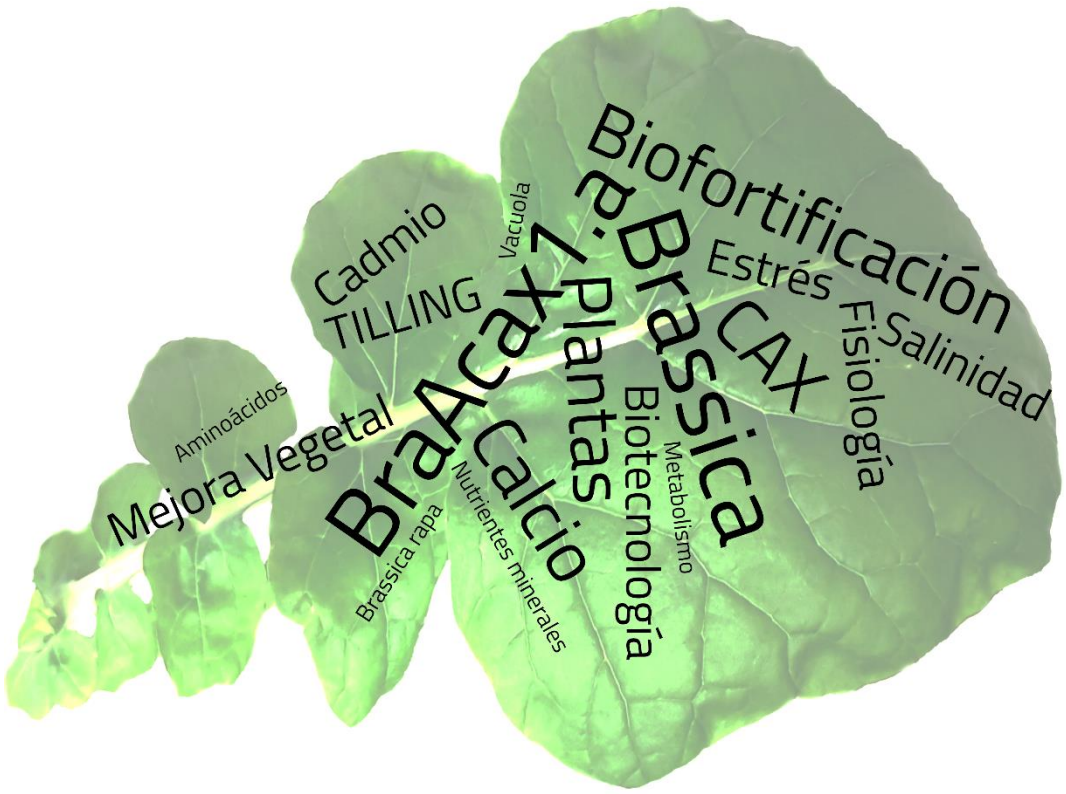
Actualmente, los niveles de salinidad están aumentando en los suelos cultivados y en las aguas de riego, lo que afecta a la producción de cultivos en todo el mundo. Este problema está presente especialmente en los cultivos de regiones áridas o semiáridas (Negrão *et al.*, 2017). Desafortunadamente, en el actual escenario de cambio climático se prevé que la salinidad de los suelos cultivados empeore en los próximos años (Gomez-Zavaglia *et al.*, 2020). La toxicidad de los iones derivada del estrés salino provoca una reducción del crecimiento de las plantas y, por consiguiente, pérdidas de rendimiento. Además, la acumulación de iones Na^+ provoca alteraciones en la fisiología de las plantas a nivel de la fotosíntesis, del potencial osmótico y causa desequilibrios nutricionales y estrés oxidativo. De hecho, una de las razones más importantes de la reducción de la biomasa en las plantas sometidas a estrés salino es la reducción del proceso de fotosíntesis, ya sea por la reducción del área foliar o por la alteración del proceso fotosintético a nivel celular (Acosta-Motos *et al.*, 2017). Otros factores negativos son la toxicidad salina directa, la reducción del suministro de CO_2 debido al cierre de los estomas o a una menor difusión, el desencadenamiento de la senescencia y la inhibición de actividades enzimáticas como la Rubisco. A corto plazo, la salinidad también causa estrés osmótico reduciendo el potencial hídrico (Parida and Das, 2005). Todos estos factores conllevan a un desequilibrio entre la actividad del PS II y el flujo de electrones. Esto conduce a una excesiva acumulación de energía fotoquímica que debe ser disipada en forma de fluorescencia o de lo contrario lleva a la acumulación de ROS que a su vez producen más daños celulares (Rangani *et al.*, 2018).

Las plantas poseen mecanismos de adaptación para hacer frente al estrés por salinidad, como por ejemplo el mantenimiento de una adecuada homeostasis iónica. Para ello es fundamental la distribución y compartimentación de los iones tóxicos, principalmente Na^+ (Acosta-Motos *et al.*, 2017). Así, las plantas limitan la absorción de Na^+ o lo acumulan en las vacuolas, para reducir sus efectos tóxicos (Wan *et al.*, 2017). Igualmente, la capacidad de las plantas para eliminar el exceso de ROS es uno de los principales factores que proporcionan tolerancia al estrés (Pokotylo *et al.*, 2012). Además, las plantas con mayor eficiencia en el uso del agua (WUE) presentan una mejor tolerancia a la salinidad y regulan mejor la pérdida de agua causada por este estrés (Parida and Das, 2005). Por otro lado, para asegurar una adecuada hidratación, las plantas deben aumentar su potencial osmótico mediante la acumulación de osmolitos. Ejemplos de osmolitos son los carbohidratos solubles como la glucosa, la sacarosa y el inositol (Acosta-Motos *et al.*, 2017). Además, un mecanismo para regular el equilibrio energético y reducir el suministro para la defensa contra el estrés es a través de la vía oxidativa de las pentosas fosfato (OPPP). En la OPPP, la glucosa-6-fosfato se oxida a pentosa-P, generando NADPH y suministrando esqueletos de carbono para la síntesis de nucleótidos, lignina, coenzimas y aminoácidos. Este proceso es impulsado por la actividad de la enzima glucosa-6-fosfato deshidrogenasa (G6PDH) (Esposito, 2016).

La participación del Ca en la tolerancia a la salinidad ha sido ampliamente comprobada. El Ca^{2+} y el Na^+ son iones antagónicos, por lo que las concentraciones tóxicas de Na^+ dificultan la función del Ca^{2+}. Además, el Ca^{2+} favorece la absorción de K^+ sobre la de Na^+ disminuyendo su efecto tóxico

(Köster *et al.*, 2018). Así pues, la homeostasis del Ca^{2+} es crucial para la tolerancia a la salinidad ya que en la respuesta al estrés, los flujos de Ca^{2+} actúan como una señal que activa los genes y las proteínas que participan en los mecanismos de tolerancia (Park *et al.*, 2016). Asimismo, se ha comprobado que el transportador CAX1 es necesario para la tolerancia a la salinidad. Este hecho se ha demostrado en plantas *cax1* que son menos resistentes a la salinidad y tienen mayores indicadores de estrés oxidativo (Pokotylo *et al.*, 2012). Además, en la halófita *S. salsa* la expresión de *CAX1* es inducida por NaCl. El transportador CAX1 de esta especie podría jugar un papel importante en la reducción de la acumulación de Ca^{2+} citosólico inducida por el estrés salino y por tanto ser fundamental en la transmisión de señales para hacer frente al estrés (Han *et al.*, 2011).

JUSTIFICACIÓN Y OBJETIVOS



1. JUSTIFICACION DEL ESTUDIO

Dada la importancia del transportador CAX1 dentro del metabolismo de la planta, a los procesos que afecta su actividad, y los problemas en agricultura a los que podría beneficiar, se justifica el estudio de mutaciones en dicho transportador. Por ello, se han generado tres mutantes distintos para el transportador CAX1 de *B. rapa* que poseen un único cambio aminoacídico (Fig. 1A). *BraA.cax1a-4*, *BraA.cax1a-7* y *BraA.cax1a-12* de *Brassica rapa* spp. R-o-18 generados por TILLING y con mutaciones en el gen CAX1 obtenidas por el grupo de investigación del Profesor Dr. Martin R. Broadley (Plant and Crop Sciences Division, University of Nottingham, UK). En un estudio previo con estos mutantes, se determinó el cambio aminoacídico que produce cada mutación y se comprobó que estos mutantes presentaban diferentes fenotipos y variaciones en las concentraciones foliares de Ca^{2+} y Mg^{2+} con respecto al parental R-o-18 (Fig. 1B) (Graham *et al.*, 2014).

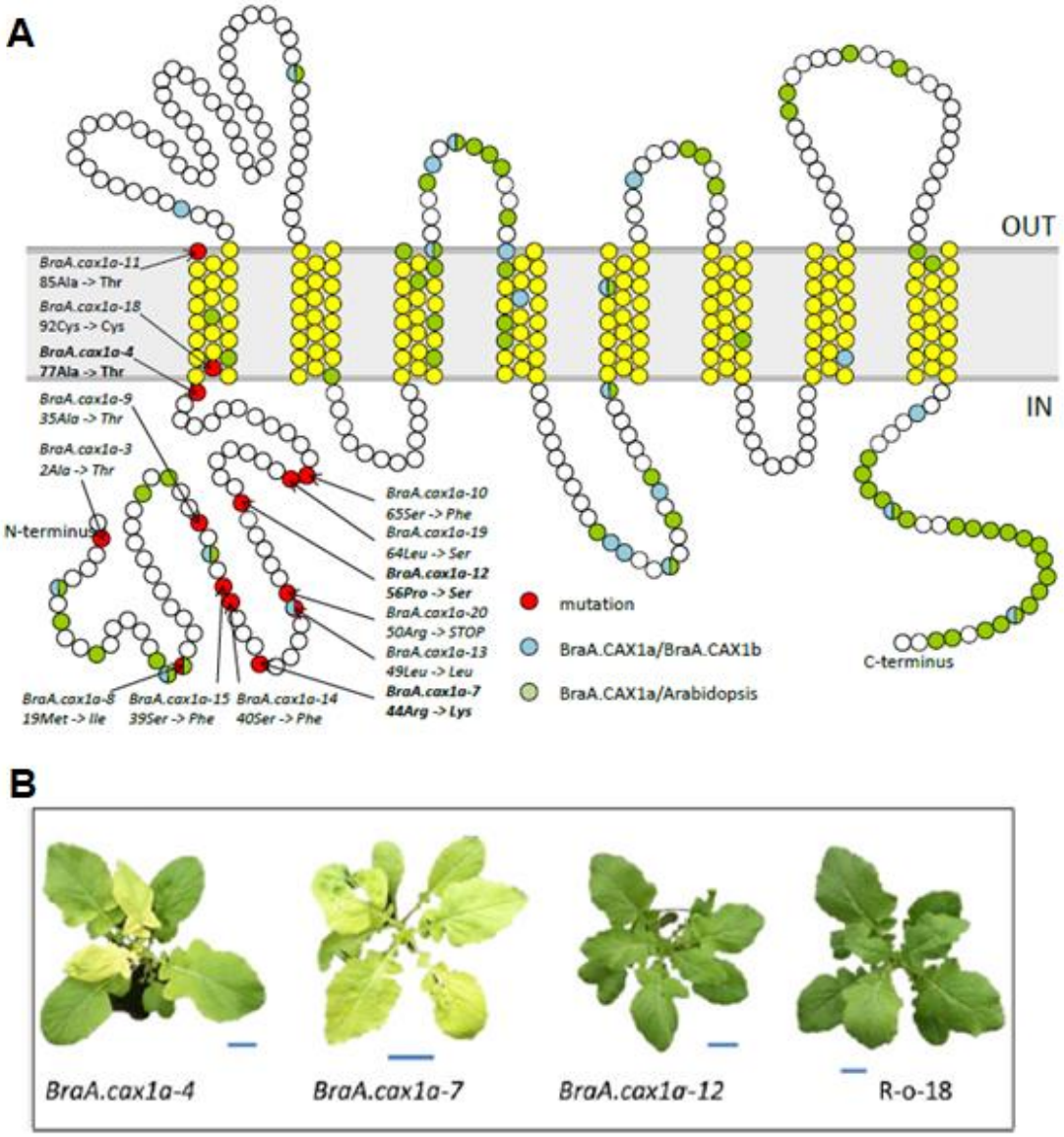


Figura 1. Representación de los cambios aminoácidos obtenidos mediante TILLING en el transportador CAX1a de *B. rapa* (A). En negrita aparecen resaltados los mutantes empleados en esta tesis. Fenotipo de los distintos mutantes *BraA.cax1a* comparado con el parental R-o-18 (B). Adaptado de (Graham *et al*., 2014).

La realización de este trabajo de investigación se justifica por los siguientes aspectos:

- Comprobar la efectividad del TILLING como técnica biotecnológica aplicable al desarrollo y mejora de la agricultura.

- Profundizar en el conocimiento existente sobre el papel que tiene CAX1 en la mejora vegetal. Se caracterizarán los efectos de mutaciones concretas en este transportador y se evaluara el potencial de los mutantes en la mejora de la acumulación de Ca^{2+} y la resistencia a distintos estreses abióticos.

- Aplicabilidad de los resultados obtenidos a la mejora vegetal de cultivos próximos a *B. rapa* de gran interés agronómico como la colza, la col, el brócoli y la coliflor.

La especie elegida para este estudio presenta un ciclo rápido, es autocompatible e incluye cultivos de hortalizas como la col china, el nabo y algunos cultivos de semillas oleaginosas (Lochlainn *et al.*, 2011). R-o-18 es una línea endogámica de la especie *B. rapa* subsp. trilocularis (Sarson amarillo) estrechamente relacionado con los cultivos de semillas oleaginosas de *B. rapa* cultivadas en Pakistán (Rana *et al.*, 2004). Fue elegida como el sistema genético para generar una población de TILLING debido a las siguientes características: 1) es diploide, lo que simplifica la genética y reduce el potencial de redundancia genética, 2) Se cuenta con un amplio conocimiento de su genoma y la información de la secuencia en el gen objetivo es un prerrequisito para la genética inversa, 3) R-o-18 se autopoliniza y produce un gran número de semillas por planta (al contrario que otras variedades de *B. rapa* como la subespecie chinensis y pekinensis) y 4) la similitud en el patrón de crecimiento tanto con la planta modelo *Arabidopsis thaliana* y las distintas variedades de colza hacen de R-o-18 un sistema adecuado tanto para la investigación fundamental, como para estudios relacionados con el rendimiento y los marcadores de calidad de *B. napus* como el manejo de su cultivo, su

arquitectura, la composición del aceite de la semilla, el desarrollo del fruto, sus nutrientes, la eficiencia en el uso del agua y la resistencia a las enfermedades (Stephenson *et al.*, 2010).

2. OBJETIVOS

El objetivo general de la presente tesis doctoral es la continuación del estudio de los tres mutantes *BraA.cax1a* centrado en la evaluación bioquímica y fisiológica con el fin de definir la función y papel del gen CAX1 en la mejora del cultivo de *B. rapa*. La utilización de dichos mutantes permitirá comprobar el papel de este gen en la mejora de la calidad de los productos agrícolas destinados al consumo humano y en la adaptación de las plantas a condiciones medioambientales adversas. Los objetivos concretos de esta Tesis doctoral consisten en el análisis del efecto de mutaciones generadas por TILLING en el transportador CAX1 de *B. rapa* spp. R-o-18 sobre los siguientes aspectos:

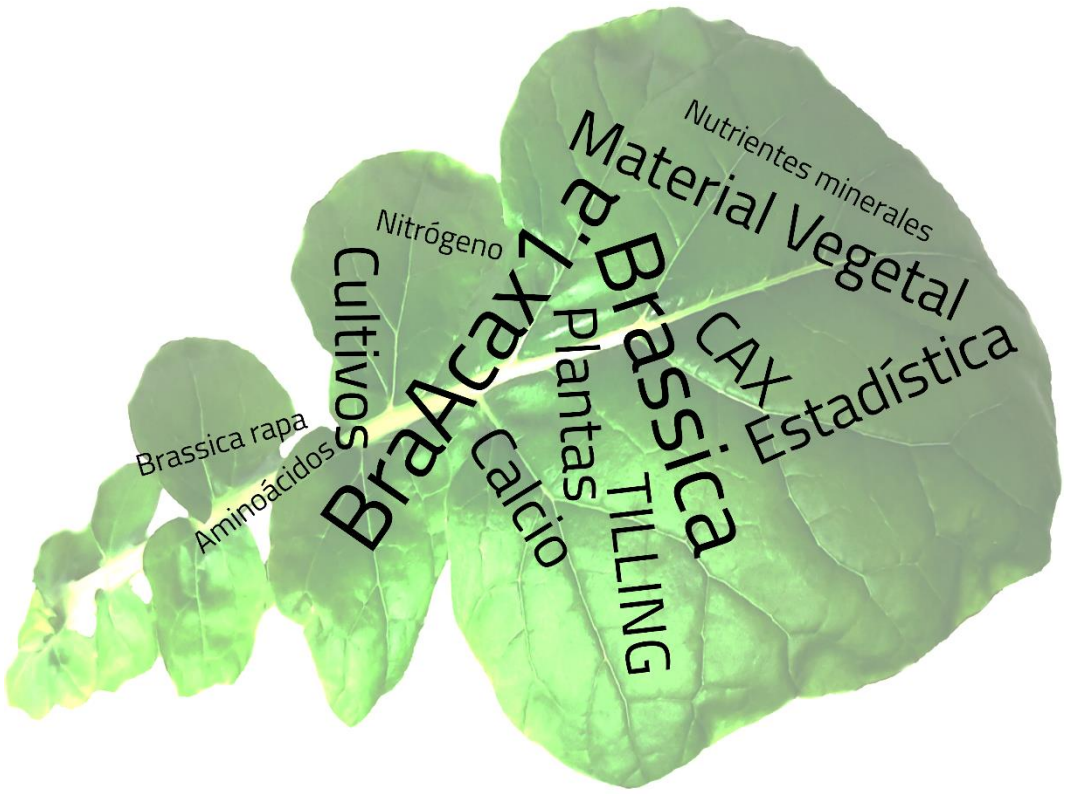
Comprobar la absorción y acumulación de Ca^{2+} a diferentes concentraciones de este catión en el medio de cultivo, evaluar la calidad nutricional y los efectos fisiológicos de cada mutación en la biofortificación con este elemento.

Comprobar la tolerancia de cada mutante frente a distintos estreses abióticos y detectar procesos clave que determinen una mayor tolerancia a dos tipos diferentes de estrés:

2.1. Toxicidad por Cd

2.2. Salinidad

MATERIAL Y MÉTODOS GENERAL



A continuación, se expone la parte común del material y métodos que se han usado para esta tesis doctoral. Información más específica se describe en los materiales y métodos de cada capítulo.

1. Material vegetal y condiciones de crecimiento

Como material vegetal para cada experimento se utilizaron tres mutantes distintos de *B. rapa* ssp. trilocularis 'R-o-18' que cuentan con un único cambio aminoacídico en su transportador CAX1a: *BraA.cax1a-4* (cambio de alanina a treonina en el aminoácido 77), *BraA.cax1a-7* (cambio de arginina a lisina en el aminoácido 44) y *BraA.cax1a-12* (cambio de prolina a serina en el aminoácido 56). Como control de dicho mutantes se emplearon plantas de la línea parental (R-o-18). Para el proceso de obtención e identificaron de las plantas mutantes se empleó la técnica de TILLING y ha sido descrito en los trabajos de Lochlainn *et al.* (2011) y Graham *et al.* (2014). Al inicio de los cultivos las semillas se sembraron en papel de filtro humedecido con agua mili-Q (18,2 MV cm) en placas de Petri de 9 cm. Las placas se mantuvieron en oscuridad durante 1 día a 4ºC antes de transferirlas a macetas llenas de vermiculita. Estas macetas se colocaron en una cámara de crecimiento bajo condiciones ambientales controladas con una humedad relativa del 60-80%, temperatura de 22/18ºC (día/noche) y un fotoperíodo de 14/10-h a una densidad de flujo de fotones fotosintéticos de 350 µmol m^{-2} s^{-1} (medida en la parte superior de las plantas con un sensor cuántico 190 SB, LI-COR Inc., Lincoln, NE, USA). A lo largo del experimento las plantas recibieron una solución de crecimiento compuesta por 4 mM KNO_3, 3 mM NH_4NO_3, 2 mM $MgSO_4$ - 7 H_2O, 6 mM KH_2PO_4, 1 mM NaH_2PO_4

- 2 H_2O, 2 µM $MnCl_2$ - 4 H_2O, 1 µM $ZnSO_4$-7H_2O, 0.25 µM $CuSO_4$ - 5 H_2O, 0.1 µM Na_2MoO_4 - 2 H_2O, 5 µM Fe-chelate (Sequestrene; 138FeG100), y 10 µM H_3BO_3. Esta solución, con un pH de 5,5-6,0, fue aplicada cada tres días.

2. Muestreo del material vegetal

Se separaron las hojas y raíces de las plantas y se lavaron con agua destilada, se secaron usando papel de filtro y se pesaron para obtener el peso fresco. Una parte del material vegetal se congeló a -40ºC para posteriormente realizar los ensayos bioquímicos y otra parte del material vegetal se liofilizó para obtener el peso seco (DW), la concentración de nutrientes y la concentración de otros compuestos de la planta.

3. Análisis de la concentración de nutrientes minerales

Las concentraciones de azufre (S), fósforo (P), potasio (K), calcio (Ca), magnesio (Mg), hierro (Fe), cobre (Cu), manganeso (Mn), zinc (Zn) y boro (B) se determinaron después de someter una muestra de 150 mg de material seco a un proceso de mineralización por digestión húmeda (Wolf, 1982). Para llevar a cabo este ensayo, las hojas secas se molieron y se mineralizaron con una mezcla de ácido nítrico (HNO_3)/ácido perclórico ($HClO_4$) (v/v) y H_2O_2 al 30%. A partir de la mineralización resultante, y tras la adición de 20 ml de H_2O mili-Q, se determinaron las concentraciones de los elementos mediante ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, EE.UU.).

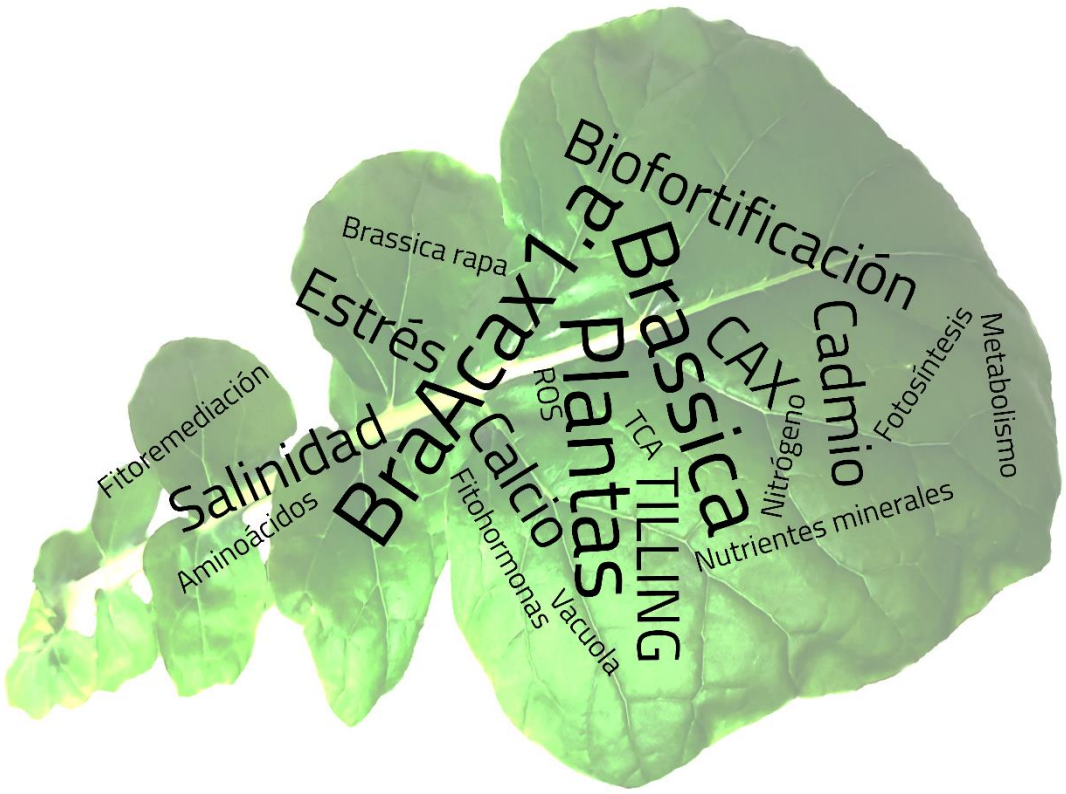
La concentración de nitrógeno total (N) se calculó como la suma de NO_3^- y de N total reducido. La concentración de NO_3^- se analizó a partir de una extracción acuosa de 0.1 g de peso seco en 10 ml de agua mili-Q. Del extracto resultante, se tomó una alícuota de 100 µl y se le añadió ácido salicílico al 10% (p/v) en ácido sulfúrico al 96% y se midió la concentración de NO_3^- por espectrofotometría según el método descrito por (Cataldo *et al.* (1975). Para la determinación del N total reducido, se realizó un mineralizado de una muestra de 0.1 g de material seco con ácido sulfúrico y H_2O_2 (Wolf, 1982). El mineralizado resultante se diluyó con agua mili-Q, y se hizo reaccionar con tartrato de potasio, hidróxido sódico y nitroprusiato sódico. La concentración de N total reducido se midió por espectrofotometría según el método descrito por (Baethgen y Alley, 1989).

4. Análisis estadístico

Los datos fueron sometidos a un análisis de ANOVA simple con un 95% de intervalo de confianza para evaluar las diferencias entre los tratamientos. Se realizó una ANOVA multifactorial para determinar si las mutaciones o los distintos tratamientos aplicados o su interacción afectaron significativamente a los resultados. Las medias fueron comparadas por el método de mínimas diferencias significativas de Fisher (LSD). Los niveles de significación para ambos análisis se expresaron como * P<0,05, ** P<0,01, *** P<0,001, o NS (no significativo). En ciertos experimentos se empleó un Análisis de Componentes Principales (ACP) para evaluar las relaciones entre los tratamientos y los

parámetros analizados. Todos los análisis estadísticos se llevaron a cabo empleando el software Statgraphics Centurión XVI.

RESULTADOS


Biofortificación
Brassica rapa
a. Brassica
Estrés
CAX
Cadmio
Fotosíntesis
Metabolismo
Fitoremediación
ROS
BraA
cax1
Plantas
Salinidad
Calcio
TCA
TILLING
Nitrógeno
Aminoácidos
Fitohormonas
Nutrientes minerales
Vacuola

CAPÍTULO 1: ESTUDIO DE LA ACUMULACIÓN DE Ca^{2+} A DIFERENTES CONCENTRACIONES DE ESTE CATIÓN EN EL MEDIO DE CULTIVO Y SUS EFECTOS FISIOLÓGICOS EN LOS TRES MUTANTES TILLING DE CAX1A DE *Brassica rapa*

1.1. Perfil fisiológico de mutantes TILLING de CAX1a de *Brassica rapa* expuestos a diferentes dosis de calcio

Resumen

El calcio (Ca) es un macronutriente esencial para las plantas y su homeostasis es básica para muchos procesos fisiológicos. Por lo tanto, tanto la deficiencia de Ca como la toxicidad constituyen problemas potenciales para los cultivos. El transportador CAX1 es un objetivo potencial para obtener plantas con una mejor homeostasis del Ca y una mayor concentración de Ca en las partes comestibles. Se obtuvieron tres mutantes de *Brassica rapa* para el transportador CAX1 mediante TILLING. El objetivo de este trabajo es evaluar el crecimiento, el estado fisiológico y la concentración de nutrientes de estos mutantes cultivados con diferentes dosis de Ca. Los mutantes y la línea parental se cultivaron con dosis bajas, controladas y altas de Ca y se determinaron parámetros relacionados con estrés oxidativo, rendimiento fotosintético y concentración de nutrientes. Los mutantes *BraA.cax1a-4* y *BraA.cax1a-7* presentaron un menor

nivel de clorofila total, un rendimiento fotosintético alterado y mayores niveles de ROS. El mutante *BraA.cax1a-12* creció mejor en condiciones de toxicidad de Ca. Todos los mutantes acumularon más Ca y Mg en las hojas bajo condiciones control de alta dosis de Ca y acumularon más Fe independientemente de la dosis de Ca. Los resultados obtenidos apuntan a *BraA.cax1a-12* como un candidato potencial para la biofortificación con Fe, Ca y Mg, ya que acumula concentraciones más altas de estos elementos, no presenta un crecimiento alterado y es capaz de tolerar mayores dosis de Ca.

Adapted from Plant Science (2018), 272: 164-172. https://doi.org/10.1016/j.plantsci.2018.04.019

Physiological profile of CAX1a TILLING mutants of *Brassica rapa* exposed to different calcium doses

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Abstract

Calcium (Ca) is an essential macronutrient for plants and its homeostasis is basic for many processes in plants. Therefore, both Ca deficiency and toxicity constitute potential issues for crops. CAX1 transporter is a potential target to obtain plants with better Ca homeostasis and higher Ca concentration in edible parts. Three *Brassica rapa* mutants for CAX1 were obtained through TILLING. The objective of this work is to evaluate the growth, physiological state and nutrients concentration of these mutants grown with different Ca doses. The mutants and the parental line were grown under low, control and high Ca doses and parameters related to their oxidative stress, photosynthetic performance and nutrients concentration were determined. *BraA.cax1a-4* and *BraA.cax1a-7* mutants presented lower total Chl, an altered photosynthesis performance and

higher ROS levels. *BraA.cax1a-12* mutant grew better under high Ca conditions. All mutants accumulated more Ca and Mg in leaves under control and high Ca doses and accumulated more Fe regardless the Ca dose. The results obtained point to *BraA.cax1a-12* as a potential candidate for biofortification with Fe, Ca and Mg since it accumulates higher concentrations of these elements, do not present an altered growth and is able to tolerate higher Ca doses.

***Key words**: Brassica rapa*, calcium, CAX1, Chl fluorescence, ionome, oxidative stress, physiological profile, TILLING.

1. Introduction

Calcium (Ca) is an essential macronutrient for plants that is present in membranes and cell walls playing a basic structure role, and in the cytosol being crucial in cell signalling processes (White and Broadley, 2003). For this reason, Ca deficiency produces serious alterations in plants and it may cost losses in crop productions. Ca deficiency can occur even whether there is an adequate supply due to low redistribution or limitations in its transport (White and Broadley, 2003; Dayod *et al.*, 2010). On the other side, Ca toxicity also reduces the plant growth rate and produce damages due to the formation of Ca oxalate crystals. Cytosolic Ca concentration must be maintained at submicromolar levels in the resting cell in order to allow rapid increases for cell signalling, which can be jeopardized by Ca toxicity (White and Broadley, 2003). Ca fluxes are also necessary in cell guards for stomata closure, so an elevated Ca concentration may promote this closure and thereby a reduction in internal CO_2 concentration and a lower

photosynthesis rate (Dayod *et al.*, 2010). Both Ca deficiency and toxicity, constitute abiotic stresses that interfere with photosynthesis impairing electron transport, decreasing photosystems efficiency, reducing photosynthetic pigments, and promoting the formation of reactive oxygen species (ROS) (Saibo *et al.*, 2009). In turn, ROS damage the photosynthetic apparatus through the disruption of thylakoid structures, inhibition of chloroplastic enzymes, and blocking PSII repair process (Gururani *et al.*, 2015).

Plants prevent Ca disorders through the regulation of plant cation/H^+ exchangers (CAXs) (Dayod *et al.*, 2010). CAXs are a family of Ca/H antiporters located on plasma and organelle membranes including vacuoles. Together with Ca-ATPases, CAXs are responsible of Ca homeostasis and Ca removal from the cytosol to generate different Ca profiles to respond environmental cues or in signalling processes (Conn *et al.*, 2011). CAXs are involved in several important aspects of plant growth and development playing a role in stomatal conductance and in pH regulation (Pittman and Hirschi, 2016). There is a strong correlation between CAXs expression and Ca accumulation. Thus, *Brassica rapa* plants have an enhanced Ca accumulation in palisade mesophyll cells (Rios *et al.*, 2012) where CAX expression is higher (Conn *et al.*, 2011). Among CAXs, CAX1 is one of the antiporters with greater Ca/H activity (Pittman and Hirschi, 2016). *CAX1* was identified as an expression quantitative trait loci that is affected by external Ca concentration in *B. rapa* (Graham *et al.*, 2014). Therefore, CAX1 is a potential target to obtain plants with better Ca homeostasis or with higher Ca concentrations in edible parts (Pittman and Hirschi, 2016). This fact could be useful since Ca, as well as iron (Fe) and magnesium (Mg) are essential elements

for human diet. Stein (2009) reported that two-thirds of the human population have a deficient diet of at least one of these elements, increasing the risk of certain diseases. Crop nutrients can be improved through two ways: providing an adequate nutrient supply in the culture medium, considering interaction between nutrients, and the other way is through crop breeding (traditional breeding and by the use of genetically modified organisms (GMOs)) (Broadley and White, 2010). Experiments have already been carried out in this regard through the expression of *Arabidopsis thaliana CAX1* (*AtCAX1*) that increased Ca concentration in vegetables such as carrots (Morris *et al.*, 2008), and lettuce (Park *et al.*, 2009). These vegetables are GMOs, i.e. they were obtained by genetic engineering techniques. A possible alternative to the use of GMOs would be the generation of mutants with a modification in CAX1 activity affecting its self-regulation or activity.

A successful approach to obtain CAX1 variants is TILLING (Targeting Induced Local Lesions In Genomes). TILLING make possible the generation and the study of allelic series of mutations in order to evaluate their effects on gene expression and in protein structure and function (Graham *et al.*, 2014). TILLING was used to generate and identify three missense mutations in *B. rapa* ssp. trilocularis 'R-o-18' Ca transporter; *BraA.CAX1a*: *BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56) (Lochlainn *et al.*, 2011). These mutations affect AAs upstream of the N-terminal autoinhibitory domain, but that could change protein conformation and thereby affecting CAX1 function or activity (Graham *et al.*, 2014). The genotyping and characterization of these

mutants has been started. *BraA.cax1a-4* and *BraA.cax1a-7* lines presented paler/yellow leaves than parental line R-o-18 and in *BraA.cax1a-7* and *BraA.cax1a-12* lines was detected a variation in Ca concentration with respect their segregant wild types (Graham *et al.*, 2014). The species chosen for this study presents a rapid cycle, is self-compatible and include vegetable crops such as Chinese cabbage, turnip and some oil-seed crops (Lochlainn *et al.*, 2011). Therefore, the working hypothesis to test is that CAX1a mutations will cause changes in growth, physiological state and nutrients accumulation and these changes will be influenced by Ca dose applied. The results could be useful to make an initial evaluation in order to improve *B. rapa* and other related crop species.

2. Material and methods

2.1. Plant material, growth conditions, and treatments

Three *B. rapa* ssp. trilocularis 'R-o-18' mutants (*BraA.CAX1a*: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12*) and the parent line R-o-18 were employed as plant material for the experiment (Lochlainn *et al.*, 2011). Seeds were sown on filter paper moistened with milli-Q water (18.2 MV cm) in 9 cm Petri dishes. The dishes were sealed with plastic film, and incubated in the dark for 1 d at 4ºC before transferring to pots filled with vermiculite. These pots where placed in a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 22/18ºC (day/night) and 14/10-h photoperiod at a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA).

Throughout the experiment the plants received a growth solution composed of 4 mM KNO_3, 3 mM NH_4NO_3, 2 mM $MgSO_4 \cdot 7\ H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4 \cdot 2\ H_2O$, 2 µM $MnCl_2 \cdot 4\ H_2O$, 0.25 µM $CuSO_4 \cdot 5\ H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2\ H_2O$, 5 µM Fe-chelate (Sequestrene; 138FeG100) and 10 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was renewed every three days.

2.2. Experimental design and treatments

Treatments were started 30 days after germination and were kept for 21 days. Plants were grown with different Ca doses: 0.4 mM of $CaCl_2$ as low Ca dose, 4 mM of $CaCl_2$ as control Ca dose, and 40 mM of $CaCl_2$ as high Ca dose. The two factors involved in the experiment were the Ca dose applied (D) and the mutant employed (M). The experimental design consisted of randomized complete block with 12 treatments, arranged in individual benches with eight plants per treatment and three replications each.

2.3. Plant sampling

Fully expanded leaves were washed with distilled water, dried on filter paper, and weighed for fresh weight (FW). Half of the leaves from each treatment were frozen at −30ºC for later biochemical assays and the other half of the plant material was lyophilized to measure dry weight (DW) and nutrient concentrations.

2.4. Analysis of mineral nutrients

Sulphur (S), phosphorus (P), potassium (K), Ca, Mg, Fe, copper (Cu), manganese (Mn), zinc (Zn) and boron (B) were determined after a sample of 150 mg dry material was subjected to a process of mineralization by wet digestion (Wolf, 1982). To carry out this assay, dry leaves were ground and mineralized with a mixture of nitric acid (HNO_3)/perchloric acid ($HClO_4$) (v/v) and H_2O_2 at 30%. From the resulting mineralization, and after the addition of 20 ml of mili-Q H_2O, elements concentrations were determined by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA). Internal standards included Sc (50 ng ml^{-1}) and Ir (5 ng ml^{-1}) in 2% TAG HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2, SPEX Certi-Prep Ltd, Stanmore, Middlesex, UK) included Al, As, Ba, Bi, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr,U, V, and Zn, in the range 0–100 µg l^{-1}, and Ca, Mg, K, and Na in the range 0–100 mg l^{-1}.

Total nitrogen (N) concentration was calculated as the sum of NO_3^- and total reduced N. NO_3^- was analysed from an aqueous extraction of 0.1 g of DW in 10 ml of Millipore-filtered water. A 100 µl aliquot was taken and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO_3^- concentration by spectrophotometry as performed by Cataldo *et al.* (1975). For total reduced N determination, a sample of 0.1 g DW was digested with sulfuric acid and H_2O_2 (Wolf, 1982). After dilution with deionized water, a 1-ml aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 µM sodium phosphate, and 5.4% w/v sodium hydroxide), 15%/0.03% (w/v) sodium silicate/sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37ºC for 15 min, and total reduced N was measured

by spectrophotometry using spectrophotometer (Infinite 200 Nanoquant, Tecan, Switzerland) according Baethgen and Alley (1989).

2.5. Ca efficiency parameters (CaUE) and distribution coefficient (DC)

CaUE parameters were calculated as follow:

Ca uptake efficiency (CaUpE) was calculated as total Ca accumulation divided by root DW (mg Ca g^{-1} RDW) (Blasco *et al.*, 2011).

Ca utilisation efficiency (CaUtE) was calculated as leaf tissue DW divided by Ca concentration (g^2 LDW mg^{-1} Ca) (Blasco *et al.*, 2011).

Distribution coefficient (DC) was calculated as the quotient between Ca concentration in leaves and Ca concentration in roots (Zhu *et al.*, 2003).

2.6. Pigment concentrations and SPAD value

Total chlorophyll (Chl) and carotenoid were extracted in methanol and centrifuged at 5000 × *g* for 5 min. Thereafter, the absorbance of the supernatant was measured at 664, 648, and 470 nm. The chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids were estimated by using the equation of Lichtenthaler (Hartmut and Lichtenthaler, 1987). Total Chl was calculated as the sum of Chl a and Chl b.

SPAD value was measured using meter SPAD-502 (Konica Minolta Sensing Inc., Japan). Three measurements were made in each leaf and average was calculated.

2.7. Chl a fluorescence analysis

Plants were adapted to dark for 30 min before measurements using a leaf clip holder that was allocated in each fully expanded leaf. Chl a fluorescence kinetics was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK); the OJIP transients were induced by red light (650 nm) with 3000 µmol photons $m^{-2}s^{-1}$ light intensity and recorded by the instrument. OJIP transients data were analysed using the JIP-test (Strasser *et al.*, 2004). Measurements were conducted with six plants of fully expanded leaves at midstem position. Parameters employed to study the energy flow and photosynthetic activities by JIP-test were: initial fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence (Fv = Fm – Fo), maximum quantum yield for primary photochemistry (Φ_{Po} = Fv/Fm), performance index (PI_{ABS}), proportion of active reaction centres (RCs) (RC/ABS), efficiency/probability with which a PSII trapped electron is transferred from Q_A to Q_B (Ψ_o), maximum quantum yield of electron transport (Φ_{Eo} = ETo/ABS) and fluorescence value at 300 µs (K step) (Strasser *et al.*, 2004).

2.8. Malondialdehyde (MDA), $O_2^{\cdot-}$, and H_2O_2 concentrations

Determination of $O_2^{\cdot -}$ in leaf extracts was based on the ability to reduce nitroblue tetrazolium (NBT) (Kubiś, 2008). Absorbance was measured at 580 nm and the $O_2^{\cdot -}$ concentration was expressed as μg g^{-1} DW.

For H_2O_2 determination leaf samples were extracted with cold acetone and the intensity of yellow colour of the supernatant was measured at 415 nm. The result of H_2O_2 concentration was expressed as μg g^{-1} DW (Kubiś, 2008).

For MDA assay, 0.1 g of leaves was homogenized in 1 ml of a 0.25% thiobarbituric acid solution in 10% trichloroacetic acid. The mixture was heated at 95 °C for 30 min and then cooled in an ice bath. Subsequently samples were centrifuged at 9500 rpm for 10 min. MDA concentration in the supernatant was quantified by measuring absorbance at 532 nm. The non-specific absorbance value at 600 nm was obtained to correct the turbidity. MDA concentration was calculated using 155 mM^{-1} cm^{-1} as extinction coefficient (Fu and Huang, 2001).

2.9. Lipoxygenase (LOX) and ascorbate peroxidase (APX) activities

LOX activity in leaf extracts was measured according to Minguez-Mosquera *et al.* (1993) using 50 mM K–phosphate buffer (pH 6.0) containing 5 mM EDTA and 1% PVP for extraction. LOX activity was calculated following the rise in the extinction at A_{234} using an extinction coefficient of 25,000 $M^{-1}cm^{-1}$.

APX activity in leaf extracts was determined by registering the absorbance change at 290 nm for 3 min of a reaction mixture containing 100 mM K-phosphate buffer (pH 7.5), 0.5 mM ascorbic acid, and 0.2 mM H_2O_2 (Rao *et al.*, 1996).

The protein concentration of extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

All spectrophotometry determinations were carried out employing spectrophotometer (Infinite 200 Nanoquant, Tecan, Switzerland).

2.10. Statistical analysis

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics Centurion XVI program. A two-tailed ANOVA was applied to ascertain whether the doses of Zn, the species, or the interaction (D * M) significantly affected the results. Means were compared by Fisher's least significant differences (LSD). The significance levels for both analyses were expressed as * P<0.05, ** P<0.01, *** P<0.001, or NS (not significant).

3. Results and discussion

3.1. Plant biomass and Ca concentration

The cause-effect link between plant biomass and Ca homeostasis may be explained through modifications in CAX1 activity. For instance, studies with *A.*

thaliana cax1 knockout mutants showed an altered plant growth and a lower foliar Ca concentration (Conn *et al.*, 2011; Pittman and Hirschi, 2016). In comparison, although the experiments employing plants with higher CAX1 activity expressing *AtCAX1* showed more shoot Ca, biomass can be reduced e.g. in tobacco (Hirschi, 1999) or not altered e.g. in carrot (Morris *et al.*, 2008) and lettuce (Park *et al.*, 2009). The results in the present work showed that *BraA.cax1a* mutations did not negatively affect to foliar biomass regardless of the Ca dose applied. However, mutants roots biomass were lower at low Ca dose and in *BraA.cax1a-4* and *BraA.cax1a-7* grown under high Ca dose (Table 1), suggesting that mutant roots are more sensitive to Ca concentration changes in the medium. Focusing on high Ca dose, *BraA.cax1a-12* mutation allowed a better growth with an 82% more leaf DW than R-o-18 plants. In the rest of lines, the application of 40 mM $CaCl_2$ caused Ca toxicity symptoms since plants showed lower foliar biomass than in control conditions (Table 1). These results coincides with the results observed by Blasco et al. (Blasco *et al.*, 2015) in which R-o-18 *B. rapa* plants also presented lower DW under high Ca dose than under a low dose. The biomass reduction may be due to Ca toxicity produces damages by the formation of Ca-oxalate crystals and interfere with Ca fluxes needed for stomata closure, causing a lower photosynthesis rate, and thereby reducing plant growth (White and Broadley, 2003; Dayod *et al.*, 2010).

Table 1. Leaf and root biomass and Ca concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

		Leaf biomass (g DW plant^{-1})	Root biomass (g DW plant^{-1})	Foliar Ca concentration (mg Ca g^{-1} DW)	Root Ca concentration (mg Ca g^{-1} DW)
0.4 mM	R-o-18	0.63ab	0.20a	10.05a	7.71b
	BraA.cax1a-4	0.61b	0.14b	9.37ab	15.05a
	BraA.cax1a-7	0.76ab	0.11c	8.98b	8.22b
	BraA.cax1a-12	0.77a	0.14b	10.03a	14.71a
	p-value	NS	***	NS	***
	$LSD_{0.05}$	0.15	0.03	0.94	1.11
4 mM	R-o-18	0.87a	0.21a	15.20c	20.91a
	BraA.cax1a-4	0.69b	0.22a	16.44b	25.24a
	BraA.cax1a-7	0.87a	0.17b	17.71a	15.34b
	BraA.cax1a-12	0.85ab	0.18b	17.29a	14.84b
	p-value	NS	**	***	**
	$LSD_{0.05}$	0.17	0.03	0.79	4.72
40 mM	R-o-18	0.34b	0.18a	28.69c	24.38a
	BraA.cax1a-4	0.40b	0.07b	40.30a	15.34b
	BraA.cax1a-7	0.37b	0.06b	43.53a	10. 05c
	BraA.cax1a-12	0.62a	0.16a	35.37b	13.48b
	p-value	***	***	***	***
	$LSD_{0.05}$	0.12	0.05	4.30	2.65
Analysis of variance					
Doses (D)		***	***	***	***
Mutation (M)		***	***	***	***
D x M		*	***	***	***
$LSD_{0.05}$		0.08	0.02	1.33	1.65

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

All *BraA.cax1a* mutations allowed a higher Ca accumulation in leaves but only when plants were grown under control and high Ca doses (Table 1). Graham *et al.* (2014) grew *BraA.cax1a* mutants with compost in a glasshouse and also observed higher Ca concentration in *BraA.cax1a-12* mutants but not in *BraA.cax1a-4* and in *BraA.cax1a-7* mutants. These differences may be due to the different culture conditions employed. In the present experiment, under low Ca

dose, mutants showed higher Ca accumulation in roots (Table 1) and a better Ca uptake capacity (Fig. 1A), although this did not result in a higher foliar Ca concentration (Table 1) since they presented a lower DC value (Fig. 1C). Therefore, mutations could enhance Ca uptake in roots but this extra Ca is not efficiently transported to the shoot. *BraA.cax1a-7* mutation reduced foliar Ca concentration but it did not decrease its foliar DW (Table 1) because it presented a higher CaUtE (Fig. 1B), i.e. this mutant may be able to improve CaUE under low Ca supply. Under control Ca supply mutant plants increased shoot Ca concentration from 8% to 16% in comparison to R-o-18 plants (Table 1) and this is due to the higher CaUpE (Fig. 1A) and higher DC values (Fig. 1C). On the other hand, *BraA.cax1a* mutations enhanced Ca uptake when high Ca dose was applied (Fig. 1A) and a great increase in its DC and foliar Ca concentration was observed (Fig. 1C; Table 1). Nevertheless, this increase in Ca concentration was moderate in *BraA.cax1a-12* (Table 1) due to a lower CaUpE (Fig. 1A) and a better CaUtE (94% higher than R-o-18; Fig. 1B) in comparison with the other mutants. This could mean that *BraA.cax1a-12* may limit Ca uptake and accumulation in order to prevent Ca toxicity which resulted in an increased leaf biomass (Table 1). Besides, *BraA.cax1a-12* CAX1 might store the Ca excess more efficiently than other mutants as it was proved that CAX1 can help to avoid Ca toxicity by storing it in vacuoles (Pittman and Hirschi, 2016).

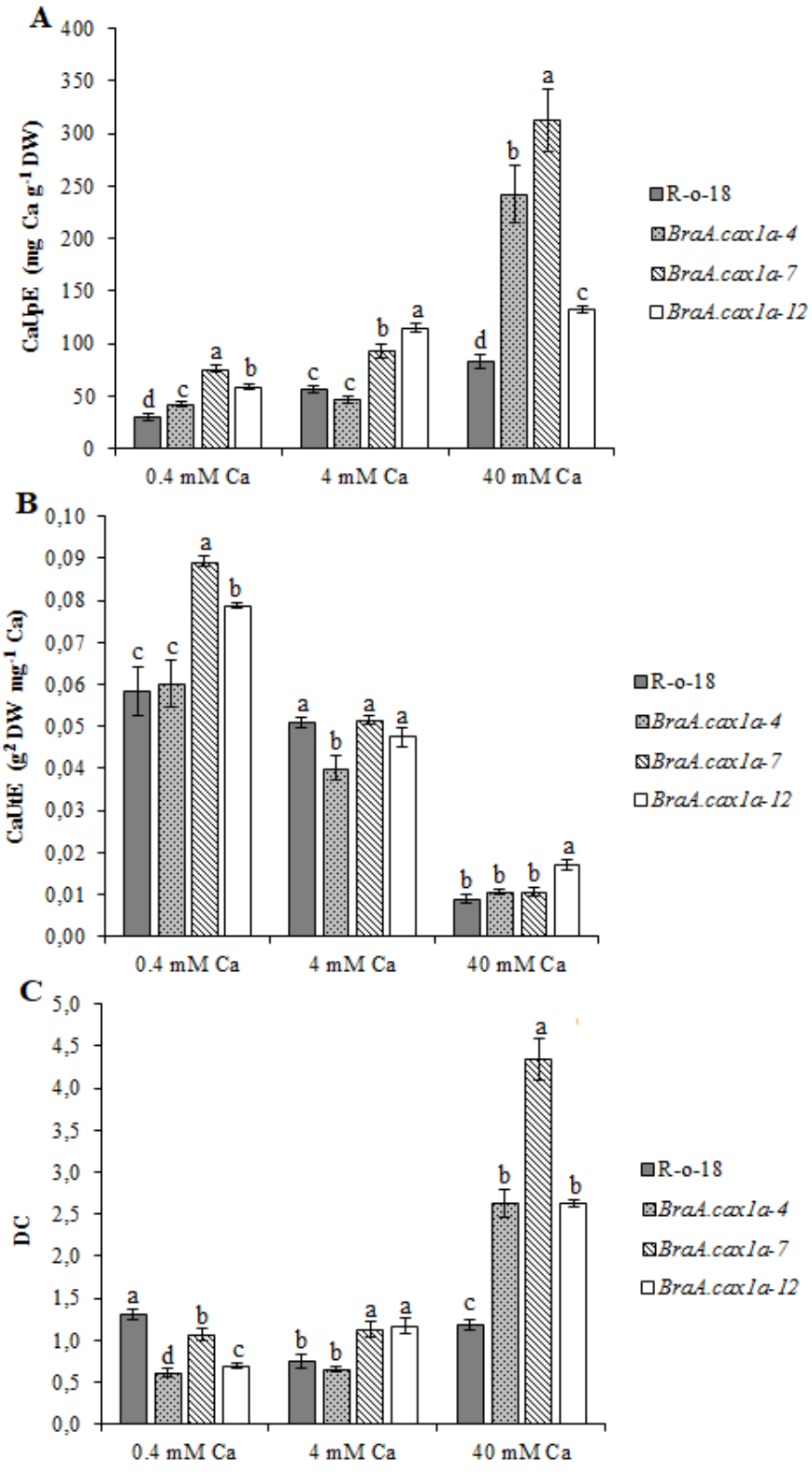

Fig. 1. Ca efficiency parameters (A and B) and DC (C) in BraA.cax1a mutants and R-o-18 plants submitted to three Ca doses. Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

3.2. Pigments concentration and photosynthetic performance

Under stress, chlorophyll content usually decreases and one method to indirectly estimate it is through SPAD meters that determine the greenness and interaction of thylakoid chlorophyll with incident light (Jifon *et al.*, 2005). In this work, *BraA.cax1a-4* and especially *BraA.cax1a-7* mutants presented lower SPAD and total Chls values in all Ca treatments in comparison to R-o-18 plants. However, total Chls concentration was slightly affected in *BraA.cax1a-12* but SPAD value was not altered (Table 2). In agreement with our results, Graham *et al.* (2014) observed leaf chlorosis in *BraA.cax1a-4* and *BraA.cax1a-7* mutants and Hirschi (Hirschi, 1999) in tobacco plant expressing *AtCAX1*. Therefore, it appears that *BraA.cax1a* mutations caused alterations in Ca homeostasis that could affect to Chls biosynthesis, chloroplast ultrastructure or photo deterioration (Xiao *et al.*, 2016). Meanwhile Chl a is mainly associated with RCs and core antenna proteins of PSII, Chl b is mainly present in light-harvesting complexes (LHCII). Thus, Chl a/b ratio is a suitable indicator of RC/ LHCII proportion. When Chl a/b is higher, this might indicate a conversion of Chl b to Chl a in order to maintain Chl a levels and thereby active RCs (Nyongesah et al., 2015). This could be occurring in mutants because they presented higher Chl a/b ratios (Table 2) suggesting a role of Ca homeostasis in Chl a/b ratio adjustment. Another important type of chloroplastic pigments are carotenoids. They are components of thylakoids that play a role as accessory light-harvesting pigments helping in the dissipation of excess energy (Havaux, 1998). Our results showed that *BraA.cax1a-7* mutation reduced carotenoids content especially under low and control Ca dose (Table 2), which might affect to photosynthetic process in this mutant.

Table 2 SPAD chlorophyll, total Chls concentration, Chl *a*/b ratio, and carotenoids concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

		SPAD value	Total Chls mg g^{-1} FW	Chl a/b	Carotenoids µg g^{-1} FW
0.4 mM	R-o-18	39.45a	0.41a	1.85c	34.47a
	BraA.cax1a-4	34.51b	0.30c	2.39a	32.34b
	BraA.cax1a-7	25.57c	0.22d	2.32b	23.23c
	BraA.cax1a-12	39.79a	0.37b	2.30b	33.52ab
	p-value	***	***	***	***
	$LSD_{0.05}$	3.60	0.01	0.06	1.19
4 mM	R-o-18	44.77a	0.41a	2.01c	36.25a
	BraA.cax1a-4	39.43b	0.34b	2.24b	29.71ab
	BraA.cax1a-7	37.16b	0.27c	2.44a	29.35b
	BraA.cax1a-12	45.27a	0.34b	2.29b	33.15ab
	p-value	***	***	***	**
	$LSD_{0.05}$	4.30	0.02	0.09	3.87
40 mM	R-o-18	54.07a	0.41a	1.93c	32.32a
	BraA.cax1a-4	45.32b	0.34b	2.24b	31.24ab
	BraA.cax1a-7	44.77b	0.27c	2.38a	29.43b
	BraA.cax1a-12	52.21a	0.33b	2.32b	30.90ab
	p-value	**	***	***	NS
	$LSD_{0.05}$	5.73	0.02	0.15	2.40
Analysis of variance					
Doses (D)		***	***	*	NS
Mutation (M)		***	***	***	***
D x M		NS	***	***	***
$LSD_{0.05}$		2.60	0.01	0.05	1.53

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

Chl a fluorescence reflects the photosynthesis status and the effects on this due to stress. When plant metabolism is disturbed, fluorescence dissipates redundant energy in order to avoid damage. Fluorescence can be quantified using the JIP test (data provided by fluorimeter) that offers parameters indicating the in vivo PS II performance (Strasser *et al.*, 2004). According JIP test results, under all Ca doses *BraA.cax1a-12* and R-o-18 plants presented similar values of fluorescence parameters except for PI_{ABS} and Ψo, Φ_{Eo} under control and high Ca

conditions that were higher in R-o-18 plants (Table 3). These results indicated that *BraA.cax1a-12* mutation only caused slight effects on photosynthesis. On the other hand, under low and control Ca dose *BraA.cax1a-4* and *BraA.cax1a-7* presented similar parameters and their values indicate an altered photosynthetic performance (Table 3). Under high Ca dose *BraA.cax1a-7* showed lower Fv/Fm, PI_{ABS}, RC/ABS, and Φ_{Eo} and higher Fo, Fm, and K step values. PI_{ABS} shows the plant capacity to resist external pressures and is a combined index that depends on RC/ABS, Fv/Fm, and Ψ_o (Strasser *et al.*, 2004). Among these factors, RC/ABS was significantly reduced in *BraA.cax1a-4* and *BraA.cax1a-7* mutants (Fig. 2). RC/ABS determines how much energy is emitted as fluorescence and how much is usable for photosynthesis (Strasser *et al.*, 2004). Therefore, the results for Fo, Fv/Fm and RC/ABS in *BraA.cax1a-4* and *BraA.cax1a-7* suggest that an impairment in Ca homeostasis caused a loss of active RCs which reduce the energy that reach to the PS II (Strasser *et al.*, 2004). Regarding Ψ_o and Φ_{Eo} parameters in *BraA.cax1a* mutants, their reduced values indicate a decrease in the electron flux through the PSII that could be caused by a decrease in quinones receptors or an accumulation of reduced quinones as suggested also by the higher Fo values (Fig. 2) (Tan *et al.*, 2011). Another parameter that usually increases under stress is K step. The higher value observed in *BraA.cax1a-4* and *BraA.cax1a-7* (Fig. 2) could indicate an uncoupling between oxygen-evolving complex (OEC) and the rest of FSII (Strasser *et al.*, 2004). Ca is part of Mn_4-Ca cluster in OEC, (Ferreira *et al.*, 2004), so in these mutants, an altered Ca homeostasis might lead to the lack of Ca for OEC and thereby affecting water oxidation and increasing K step (Table 3).

Table 3 Values of Chl a fluorescence parameters derived from the JIP test in *BraA.cax1a* mutants and R-o-1 doses

		Fo	Fm	Fv	Fv/Fm	PI_{ABS}	RC/ABS
0.4 mM	R-o-18	249b	1615ab	1366a	0.85a	8.68a	0.85a
	BraA.cax1a-4	299a	1690a	1390a	0.82b	5.11c	0.67b
	BraA.cax1a-7	273ab	1473b	1200b	0.82b	4.27c	0.64b
	BraA.cax1a-12	252b	1582ab	1330a	0.84a	7.15b	0.78a
	p-value	NS	*	**	**	***	***
	$LSD_{0.05}$	43	140	104	0.01	1.46	0.08
4 mM	R-o-18	252b	1660b	1408b	0.85a	10.30a	0.89a
	BraA.cax1a-4	329a	1800a	1471a	0.82b	6.27c	0.73b
	BraA.cax1a-7	343a	1841a	1498a	0.81b	5.49c	0.73b
	BraA.cax1a-12	262b	1683b	1421b	0.84a	8.77b	0.84a
	p-value	**	**	**	***	***	**
	$LSD_{0.05}$	52.46	92	50	0.01	1.76	0.09
40 mM	R-o-18	250b	1668b	1418ab	0.85a	15.72a	1.02a
	BraA.cax1a-4	294b	1675b	1381b	0.82b	7.27c	0.85b
	BraA.cax1a-7	422a	1965a	1544a	0.79c	5.33d	0.77c
	BraA.cax1a-12	264b	1702b	1438ab	0.85a	12.53b	1.06a
	p-value	***	**	NS	***	***	***
	$LSD_{0.05}$	55	182	131	0.01	1.50	0.07
Analysis of variance							
Doses (D)		**	**	**	NS	***	***
Mutation (M)		***	***	***	***	***	***
D x M		***	***	**	***	***	*
$LSD_{0.05}$		28	76	56	0.01	0.87	0.05

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.
significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (

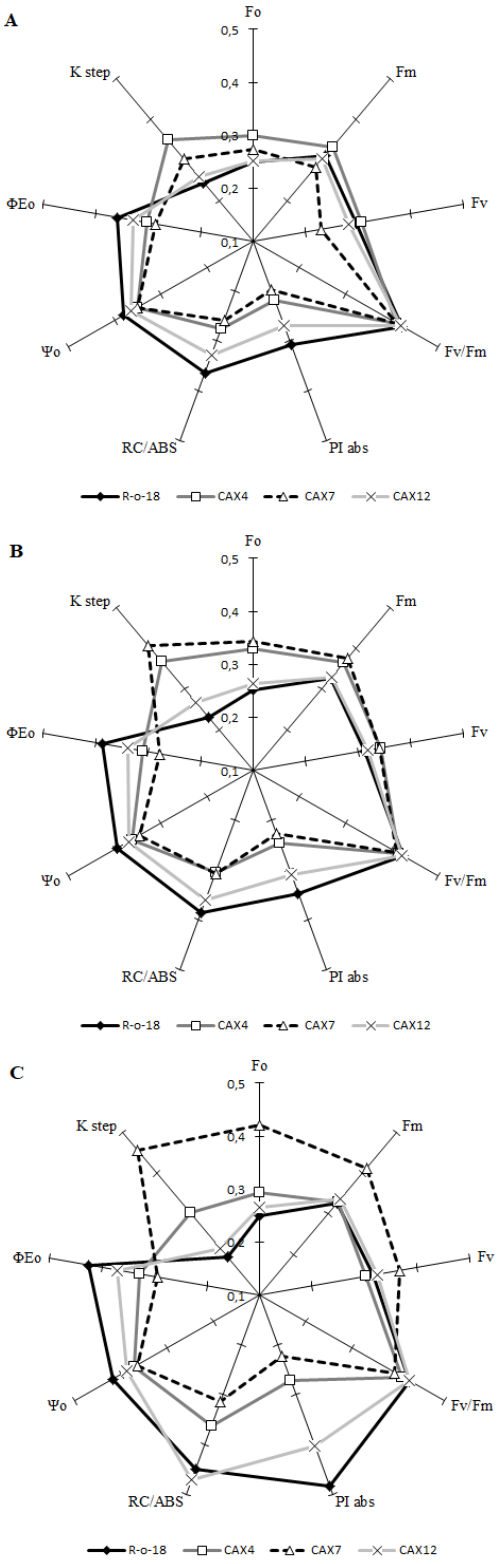

Fig. 2. Values of Chl a fluorescence parameters derived from the JIP test in *BraA.cax1a* mutants and R-o-18 plants submitted to 0.4 mM $CaCl_2$ (A), 4 mM $CaCl_2$ (B), and 40 mM $CaCl_2$ (C). Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

Lang *et al.* (1996) observed that the reduction in Chl a and carotenoids concentration affected negatively photosynthesis and promoted a Chl fluorescence rise. This was observed especially in *BraA.cax1a-7* grown under high Ca conditions (Table 2 and Fig. 2C). In this case, light energy is emitted as fluorescence instead of being absorbed in RCs and light-harvesting complexes could be reorganized in order to maximize the dissipation of excess energy produced by stress (Lang *et al.*, 1996). Tan *et al.*, (2011) observed that exogenous Ca application can protect photosynthesis against stress improving Ψ_o and PI_{ABS} parameters. Ca helps to repair the PSII complex, maintain photosynthesis and activates antioxidant enzymes. The latter is important since ROS cause photodamage to PSII and inhibits its reparation (Gururani *et al.*, 2015). Besides, Ca can directly active antioxidant enzymes to eliminate ROS dissipating excess energy and preventing photodamage (Tan *et al.*, 2011).

3.3. Oxidative stress

In the present study MDA, O_2^-, H_2O_2 concentrations and LOX activity were analysed as oxidative-stress indicators. The higher MDA and O_2^- concentrations and LOX activity in *BraA.cax1a-4* and *BraA.cax1a-7* plants indicated that they were the mutants that showed higher oxidative stress (Table 4). In these mutant plants, ROS could react especially with unsaturated fatty acids producing MDA as a subproduct of lipid peroxidation. Likewise, this could be strengthened by the increase in LOX activity that, in turn, promotes O_2^- formation (Karuppanapandian *et al.*, 2011). Pokotylo *et al.* (2012) showed that an altered *CAX1* expression can lead to a higher oxidative stress as observed in *cax1* tobacco mutants that presented higher MDA levels and lower activities of antioxidant enzymes.

Therefore the higher oxidative stress in *BraA.cax1a-4* and *BraA.cax1a-7* probably was caused by alterations in Ca homeostasis and because Ca affects ROS concentration since both are involved in cell signalling to stress (Bowler and Fluhr, 2000). On the other hand, Gururani *et al.* (2015) observed that ROS adversely affect the photosynthetic system and total Chl reduction can be an indicator of damage caused by ROS. Accordingly, the higher oxidative stress observed in *BraA.cax1a-4* and *BraA.cax1a-7* mutants (Table 4) might be responsible for their altered photosynthetic performance (Fig. 2). To prevent the oxidative damage, antioxidant compounds in chloroplasts such as carotenoids prevent and eliminate ROS accumulation (Havaux, 1998). Thus, in our study, the results for O_2^- (Table 4), total Chls and carotenoids (Table 2) suggested that *BraA.cax1a-7* mutant presented the higher oxidative stress and lower antioxidant capacity under low and control conditions. In contrast, under high Ca conditions, *BraA.cax1a-7* registered lower MDA and LOX levels (Table 4). Hence, in this mutant high Ca dose might not affect to the antioxidant machinery that protect against MDA formation.

Table 4 Values of O_2^-, H_2O_2, and MDA concentrations, and APX and LOX activities in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

		MDA (μM g^{-1} FW)	LOX (ΔAbs mg $prot^{-1}min^{-1}$)	O_2^- (μg g^{-1} FW)	H_2O_2 (μg g^{-1} FW)	APX (ΔAbs mg $prot^{-1}min^{-1}$)
0.4 mM	R-o-18	10.64b	7.74b	6.08bc	0.41a	0.23c
	BraA.cax1a-4	12.49a	7.66ab	6.30b	0.34b	0.37b
	BraA.cax1a-7	12.65a	8.36a	7.68a	0.29c	0.50a
	BraA.cax1a-12	11.94ab	6.18b	5.56c	0.29c	0.55a
	p-value	NS	*	***	***	***
	$LSD_{0.05}$	1.63	1.62	0.66	0.03	0.09
4 mM	R-o-18	19.14c	6.07b	3.67c	0.34a	0.20c
	BraA.cax1a-4	28.64a	10.85a	4.66b	0.28b	0.36b
	BraA.cax1a-7	22.77b	10.90a	6.67a	0.26b	0.48a
	BraA.cax1a-12	18.92c	7.82 b	3.76c	0.27b	0.41ab
	p-value	***	***	***	*	***
	$LSD_{0.05}$	1.36	1.89	0.42	0.06	0.10
40 mM	R-o-18	19.59b	10.43b	3.78b	0.37b	0.35c
	BraA.cax1a-4	32.85a	13.41a	4.80a	0.32c	0.60a
	BraA.cax1a-7	10.49d	7.08c	4.72a	0.42a	0.57a
	BraA.cax1a-12	12.24c	8.18c	3.88b	0.28d	0.49b
	p-value	***	***	***	***	***
	$LSD_{0.05}$	1.22	1.87	0.33	0.03	0.08
Analysis of variance						
Doses (D)		***	***	**	***	***
Mutation (M)		***	***	***	***	***
D x M		***	***	***	***	***
$LSD_{0.05}$		0.79	1.05	0.28	0.02	0.05

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

Plants possess mechanisms to eliminate ROS through antioxidant enzymes such as APX activity that is an efficient H_2O_2 scavenger (Du *et al.*, 2001). In the present work, under low and control Ca doses mutants registered lower H_2O_2 values and higher APX activity in comparison to R-o-18. Blasco *et al.* (2015) in *B. rapa* plants submitted to Ca toxicity also observed an increase in APX activity.

These results suggest that APX is an important enzyme in ROS elimination in this species. On the other hand, some studies proved that Ca applied at the proper dose can be beneficial to reduce oxidative stress (Tan *et al.*, 2011). La aplicación de Ca en la dosis adecuada puede ser beneficiosa para reducir el estrés oxidativo (Tan et al., 2011). Likewise, in the present study *BraA.cax1a* mutations produced an improved H_2O_2 detoxification through APX enzyme. One exception is *BraA.cax1a-7* plants submitted to high Ca dose where the higher APX activity was not efficient enough to reduce H_2O_2 concentration because its concentration was higher than in R-o-18 plants. Nevertheless *BraA.cax1a-4* and *BraA.cax1a-7* presented higher O_2^- levels than R-o-18 (Table 4) and this could explain the altered photosynthesis performance (Fig. 2) since O_2^- is the ROS with the highest oxidant power and thereby more dangerous than H_2O_2 (Karuppanapandian *et al.*, 2011).

3.4. Nutrient concentrations

Several studies carried out on plants with modified *CAX1* expression reported altered accumulations of mineral nutrients (Hirschi, 1999; Conn *et al.*, 2011). In the present study, *BraA.cax1a-12* mutation caused a higher N and S accumulations regardless the Ca dose applied (Fig. 3A-B). N has a direct relationship with plant biomass (Ruiz and Romero, 1999) and S is an important component of glutathione, hormones, and certain amino acids (Scherer, 2001). Therefore, this higher N and S concentrations may help *BraA.cax1a-12* to maintain growth, especially when high Ca doses are applied (Table 1). In addition, S deficiency limits yield in crops all over the world (Scherer, 2001), so

BraA.cax1a-12 mutation could be useful under S deficiency conditions (Fig. 3C). With respect P concentration, only slight differences were found between mutants and R-o-18 plants, highlighting a higher P accumulation in mutants under control Ca dose (Fig. 3C). This may be due to the relationship between CAX1 activity and P absorption observed by Liu *et al.* (2011). Regarding Mg, its concentration increased when both low and high Ca dose were applied in mutant plants in comparison to control Ca dose; whereas in comparison to R-o-18 all mutants accumulated more Mg under control and high Ca doses (Fig. 3E). In previous experiments in *B. rapa* plants authors observed the well-known negative interaction between Ca and Mg (Rios *et al.*, 2012; Blasco *et al.*, 2015). Nevertheless, in the present work this relation was not observed in *BraA.cax1a* mutants when high Ca dose was applied (Fig. 3E). One possibility is that the extra Ca is being stored in vacuoles. The higher Mg accumulation may be beneficial to counteract the higher Ca concentration that could be toxic especially in high Ca treatment.

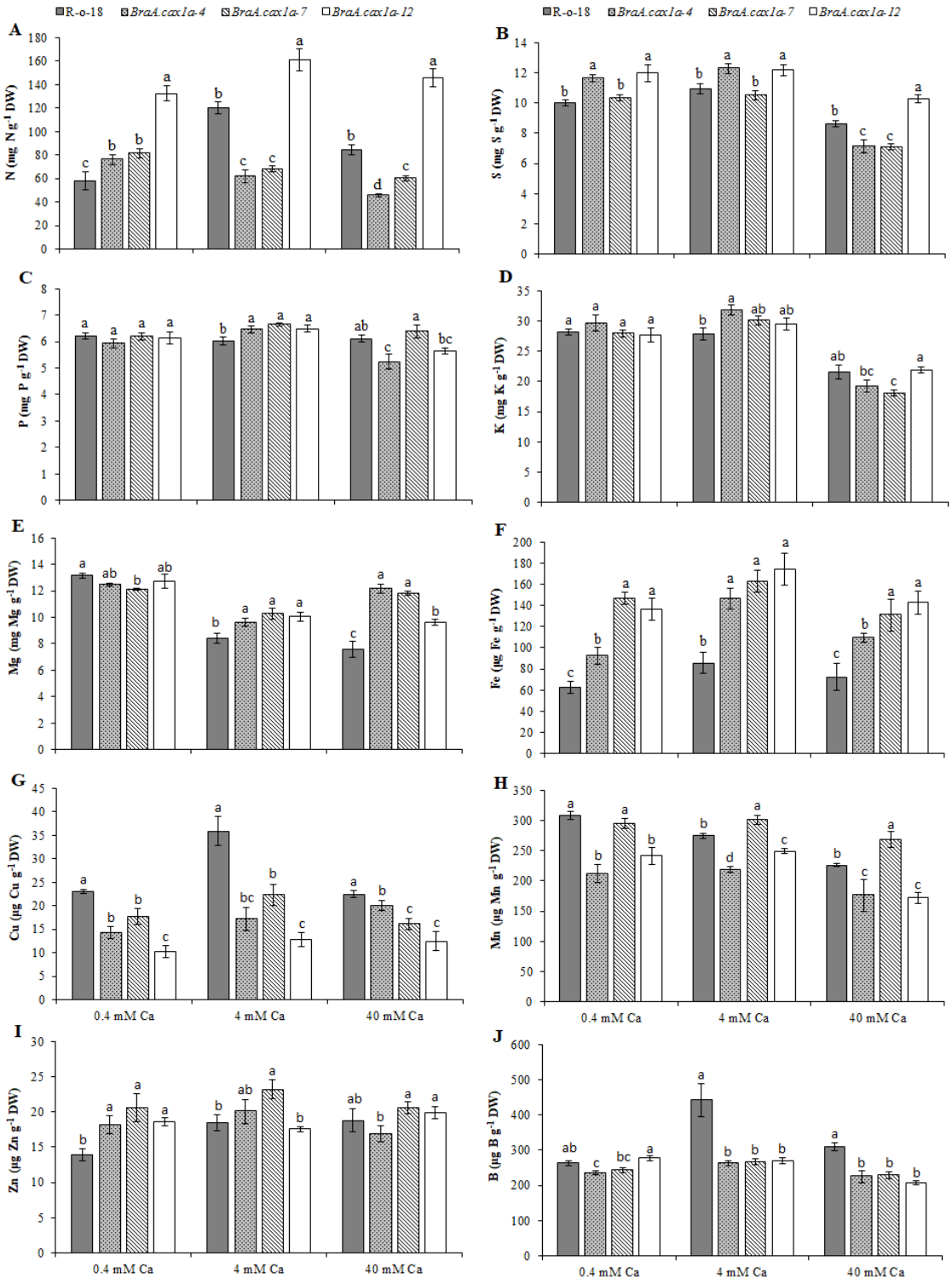

Fig. 3. Nutrient concentrations of *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses. Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

Micronutrients are required in lower concentrations but play a key role in numerous processes in plants (Marschner, 2011). In the present study, *BraA.cax1a* mutations enhanced Fe accumulation disregarding the Ca dose applied (Fig. 3F) while the accumulation of Cu, Mn and B generally decreased in comparison to R-o-18 plants (Fig. 3G-J). A higher Fe accumulation might explain the higher APX activity in *BraA.cax1a* mutants (Table 4) since. Vansuyt *et al.* (1997) proved that this element is necessary for APX activation. On the other hand, the antagonistic relationship between Ca and B (Gupta and Macleod, 1981) might be boosted in *BraA.cax1a* mutants and reduce B accumulation in leaves (Fig. 3J). Finally, regarding Mn, *BraA.cax1a-4* and *BraA.cax1a-12* reduced its accumulation in leaves (Fig. 3H). Conn *et al.* (2011) observed that besides Ca, CAX1 can also transport Mn. Therefore, the CAX1 transporter of *BraA.cax1a-4* and *BraA.cax1a-12* mutants might have a lower Mn transport capacity that may limit its accumulation in leaves.

In addition to the importance of nutrients for the normal plant growth and development, it is interesting to study its accumulation from the human nutrition point of view (Broadley and White, 2010). Thus, the results suggest that *BraA.cax1a* mutants could be employed in Fe biofortification programs regardless the Ca dose applied, especially *BraA.cax1a-12* that reached increases in its Fe concentration around double of R-o-18 (Fig. 3F). However, in the case of other nutrients, their accumulation depends on the Ca dose applied, so biofortification programs efficiency could be enhanced through Ca managing. Thereby, Ca and Mg only are accumulated under control and high Ca doses (Table 1 and Fig. 3E), whereas Zn only is accumulated under low Ca dose (Fig.

3I). Therefore, the Ca dose supplied to the plant is a key factor to be considered in biofortification program with *BraA.cax1a* mutants.

4. Conclusions

The results obtained proved the working hypothesis because CAX1a mutations caused changes in growth, physiological state and nutrients accumulation and these changes were influenced by Ca dose applied. *BraA.cax1a* mutants appear to be unhelpful to better Ca-deficiency tolerance since they did not improve plant growth neither foliar Ca accumulation under low Ca application. In addition, regardless the Ca dose applied, *BraA.cax1a-4* and *BraA.cax1a-7* showed stress symptoms such as lower total Chl, an altered photosynthesis performance and higher ROS levels. However, *BraA.cax1a-12* did not affect negatively to plant vitality. Indeed, this mutation allows a better growth under high Ca conditions. All mutants accumulated more Ca and Mg in leaves when Ca is in adequate concentration in the medium and accumulated more Fe regardless the Ca dose. These results obtained point to *BraA.cax1a-12* as a potential candidate for biofortification with Fe, Ca and Mg since it accumulates these elements to a greater extent than R-o-18, does not present an altered growth and is able to tolerate higher Ca doses. Besides this mutant might be employed for phytoremediation purposes in soils with toxic concentrations of these elements, although specific studies are required.

Acknowledgments

This work was financed by the PAI programme (Plan Andaluz de Investigación, Grupo de Investigación AGR161) and by a Grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL [FPU14/01858].

References

Baethgen, W.E., Alley, M.M., 1989. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. Commun. Soil Sci. Plant Anal. 20, 961–969. https://doi.org/10.1080/00103628909368129

Blasco, B., Graham, N.S., Broadley, M.R., 2015. Antioxidant response and carboxylate metabolism in *Brassica rapa* exposed to different external Zn, Ca, and Mg supply. J. Plant Physiol. 176, 16–24. https://doi.org/10.1016/j.jplph.2014.07.029

Blasco, B., Rios, J.J., Cervilla, L.M., Sánchez-Rodríguez, E., Rubio-Wilhelmi, M.M., Rosales, M.A., Romero, L., Ruiz, J.M., 2011. Iodine application affects nitrogen-use efficiency of lettuce plants (*Lactuca sativa* L.). Acta Agric. Scand. Sect. B — Soil Plant Sci. 61, 378–383. https://doi.org/10.1080/09064710.2010.492782

Bowler, C., Fluhr, R., 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant Sci. 5, 241–246. https://doi.org/10.1016/S1360-1385(00)01628-9

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Broadley, M.R., White, P.J., 2010. Eats roots and leaves. Can edible horticultural crops address dietary calcium, magnesium and potassium deficiencies? Proc. Nutr. Soc. 69, 601–12. https://doi.org/10.1017/S0029665110001588

Cataldo, D.A., Maroon, M., Schrader, L.E., Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6, 71–80. https://doi.org/10.1080/00103627509366547

Conn, S.J., Gilliham, M., Athman, A., Schreiber, A.W., Baumann, U., Moller, I., Cheng, N.-H., Stancombe, M.A., Hirschi, K.D., Webb, A.A.R., Burton, R., Kaiser, B.N., Tyerman, S.D., Leigh, R.A., 2011. Cell-specific vacuolar calcium

storage mediated by CAX1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis*. Plant Cell 23, 240–257. https://doi.org/10.1105/tpc.109.072769

Dayod, M., Tyerman, S.D., Leigh, R.A., Gilliham, M., 2010. Calcium storage in plants and the implications for calcium biofortification. Protoplasma 247, 215–231. https://doi.org/10.1007/s00709-010-0182-0

Du, X.M., Yin, W.X., Zhao, Y.X., Zhang, H., 2001. The production and scavenging of reactive oxygen species in plants. Chin. J. Biotechnol. 17, 121–5

Ferreira, K.N., Iverson, T.M., Maghlaoui, K., Barber, J., Iwata, S., 2004. Architecture of the photosynthetic oxygen-evolving center. Science 303(5665), 1831-1838.

Fu, J., Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45, 105–114. https://doi.org/10.1016/S0098-8472(00)00084-8

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of *Brassica* under altered Ca supply identifies *Arabidopsis* Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Gupta, U., Macleod, J., 1981. Plant and soil boron as influenced by soil pH and calcium sources on podzol soils. Soil Sci. 131, 20

Gururani, M.A., Venkatesh, J., Tran, L.S.P., 2015. Regulation of photosynthesis during abiotic stress-induced photoinhibition. Mol. Plant 8, 1304–1320. https://doi.org/10.1016/j.molp.2015.05.005

Hartmut, A., Lichtenthaler, K., 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382. https://doi.org/10.1016/0076-6879(87)48036-1

Havaux, M., 1998. Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci. 3, 147–151. https://doi.org/10.1016/S1360-1385(98)01200-X

Hirschi, K.D., 1999. Expression of Arabidopsis CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell 11, 2113–2122. https://doi.org/10.1105/tpc.11.11.2113

Jifon, J.L., Syvertsen, J.P., Whaley, E., 2005. Growth environment and leaf anatomy affect nondestructive estimates of chlorophyll and nitrogen in *Citrus* sp. leaves. J. Am. Soc. Hortic. Sci. 130, 152–158

Karuppanapandian, T., Moon, J.C., Kim, C., Manoharan, K., Kim, W., 2011. Reactive oxygen species in plants: Their generation, signal transduction, and scavenging mechanisms. Aust. J. Crop Sci. 5, 709–725

Kubiś, J., 2008. Exogenous spermidine differentially alters activities of some scavenging system enzymes, H_2O_2 and superoxide radical levels in water-stressed cucumber leaves. J. Plant Physiol. 165, 397–406. https://doi.org/10.1016/j.jplph.2007.02.005

Lang, M., Lichtenthaler, H.K., Sowinska, M., Heisel, F., Miehé, J.A., 1996. Fluorescence imaging of water and temperature stress in plant leaves. J. Plant Physiol. 148, 613–621. https://doi.org/10.1016/s0176-1617(96)80082-4

Liu, T.-Y., Aung, K., Tseng, C.-Y., Chang, T.-Y., Chen, Y.-S., Chiou, T.-J., 2011. Vacuolar Ca^{2+}/H^{+} transport activity is required for systemic phosphate homeostasis involving shoot-to-root signaling in *Arabidopsis*. Plant Physiol. 156, 1176–1189. https://doi.org/10.1104/pp.111.175257

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R., 2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

Marschner, 2011. Marschner's Mineral Nutrition of Higher Plants, Petra Mars. ed. Academic Press

Minguez-Mosquera, M.I., Jaren-Galan, M., Garrido-Fernandez, J., 1993. Lipoxygenase activity during pepper ripening and processing of paprika. Phytochemistry 32, 1103–1108. https://doi.org/10.1016/S0031-9422(00)95073-8

Morris, J., Hawthorne, K.M., Hotze, T., Abrams, S. a, Hirschi, K.D., 2008. Nutritional impact of elevated calcium transport activity in carrots. Proc. Natl. Acad. Sci. U. S. A. 105, 1431–5. https://doi.org/10.1073/pnas.0709005105

Nyongesah, M.J., Wang, Q., Li, P., 2015. Effectiveness of photochemical reflectance index to trace vertical and seasonal chlorophyll a/b ratio in *Haloxylon*

ammodendron. Acta Physiol. Plant. 37, 2. https://doi.org/10.1007/s11738-014-1747-x

Park, S., Elless, M.P., Park, J., Jenkins, A., Lim, W., Chambers IV, E., Hirschi, K.D., 2009. Sensory analysis of calcium-biofortified lettuce. Plant Biotechnol. J. 7, 106–117. https://doi.org/10.1111/j.1467-7652.2008.00379.x

Pittman, J.K., Hirschi, K.D., 2016. CAX-ing a wide net: Cation/H^+ transporters in metal remediation and abiotic stress signaling. Plant Biol. 18, 741–749. https://doi.org/10.1111/plb.12460

Pokotylo, I. V, Kretinin, S. V, Kravets, V.S., 2012. Role of phospholipase D in metabolism reactions of transgenic tobacco *cax1* cells under salinity stress. Cytol. Genet. 46, 3–9. https://doi.org/10.3103/S0095452712030085

Rao, M. V, Paliyath, G., Ormrod, D.P., 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol. 110, 125–36. https://doi.org/10.1104/PP.110.1.125

Rios, J.J., Lochlainn, S.O., Devonshire, J., Graham, N.S., Hammond, J.P., King, G.J., White, P.J., Kurup, S., Broadley, M.R., 2012. Distribution of calcium (Ca) and magnesium (Mg) in the leaves of *Brassica rapa* under varying exogenous Ca and Mg supply. Ann. Bot. 109, 1081–1089. https://doi.org/10.1093/aob/mcs029

Ruiz, J.M., Romero, L. 1999. Cucumber yield and nitrogen metabolism in response to nitrogen supply. Sci. Hortic. 82(3-4), 309–316.

Saibo, N.J.M., Lourenço, T., Oliveira, M.M., 2009. Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Ann. Bot. 103, 609–23. https://doi.org/10.1093/aob/mcn227

Scherer, H.W., 2001. Sulphur in crop production. Eur. J. Agron. 14, 81–111. https://doi.org/10.1016/S1161-0301(00)00082-4

Stein, A.J., 2009. Global impacts of human mineral malnutrition. Plant Soil 335, 133–154. https://doi.org/10.1007/s11104-009-0228-2

Strasser, R.J., Tsimilli-Michael, M., Srivastava, A., 2004. Analysis of the Chlorophyll a Fluorescence Transient, in: Chlorophyll a Fluorescence. Springer Netherlands, Dordrecht, pp. 321–362. https://doi.org/10.1007/978-1-4020-3218-9_12

Tan, W., Meng, Q. wei, Brestic, M., Olsovska, K., Yang, X., 2011. Photosynthesis is improved by exogenous calcium in heat-stressed tobacco plants. J. Plant Physiol. 168, 2063–2071. https://doi.org/10.1016/j.jplph.2011.06.009

Vansuyt, G., Lopez, F., Inzé, D., Briat, J.-F., Fourcroy, P., 1997. Iron triggers a rapid induction of ascorbate peroxidase gene expression in *Brassica napus*. FEBS Lett. 410, 195–200. https://doi.org/10.1016/S0014-5793(97)00587-5

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511. https://doi.org/10.1093/aob/mcg164

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Xiao, F., Yang, Z.Q., Lee, K.W., 2016. Photosynthetic and physiological responses to high temperature in grapevine (*Vitis vinifera* L.) leaves during the seedling stage. J. Hortic. Sci. Biotechnol. 92, 2–10. https://doi.org/10.1080/14620316.2016.1211493

Zhu, Y.-G., Huang, Y.-Z., Hu, Y., Liu, Y.-X., 2003. Iodine uptake by spinach (*Spinacia oleracea* L.) plants grown in solution culture: effects of iodine species and solution concentrations. Environ. Int. 29, 33–37. https://doi.org/10.1016/S0160-4120(02)00129-0

1.2. Efecto de mutaciones TILLING en CAX1a y de la concentración de calcio en algunos procesos del metabolismo primario en plantas de *Brassica rapa*

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Resumen

Los transportadores de intercambio catiónico/H^+ (CAX) son cruciales en la homeostasis del Ca^{2+} y en la generación de los perfiles de Ca^{2+} que participan en los procesos de señalización. Dado el papel crucial del CAX1 en la homeostasis del Ca^{2+}, las modificaciones de CAX1 podrían tener efectos en el metabolismo de las plantas. Tres mutantes de *Brassica rapa* para CAX1 se obtuvieron a través de TILLING. El objetivo de este trabajo es evaluar el efecto de las diferentes mutaciones y las diferentes dosis de Ca^{2+} en el metabolismo de las plantas. Para ello, se cultivaron los mutantes y la línea parental bajo tres dosis de Ca^{2+} (baja, control y alta) y se midieron parámetros relacionados con el metabolismo del nitrógeno (N), el ciclo de los ácidos tricarboxílicos (TCA), y se midieron los perfiles de aminoácidos (AAs) y fitohormonas. Los resultados mostraron que las mutaciones de *BraA.cax1a* afectan al metabolismo, especialmente en dosis altas de Ca^{2+}. Así, *BraA.cax1a-7* inhibió algunas enzimas del metabolismo N y activó la actividad fotorrespiratoria. Por otro lado, la mutación *BraA.cax1a-12* proporciona una mejor tolerancia a las dosis altas de

Ca^{2+}. Esta tolerancia podría ser proporcionada por una mejora de las enzimas del metabolismo N y TCA, y una mayor concentración de glutamato, malato, ácido indol-3-acético y ácido abscísico. Por lo tanto, la mutación *BraA.cax1a-12* podría utilizarse para mejorar *B. rapa* y los cambios metabolómicos observados en este mutante podrían ser responsables de una mejor tolerancia a dosis altas de Ca^{2+}.

Adapted from Journal of Plant Physiology (2019), 237: 51-60. https://doi.org/10.1016/j.jplph.2019.04.002

Effect of CAX1a TILLING mutations and calcium concentration on some primary metabolism processes in *Brassica rapa* plants

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Abstract

Cation/H^+ exchanger transporters (CAXs) are crucial in Ca^{2+} homeostasis and in the generation of Ca^{2+} profiles involved in signalling processes. Given the crucial role of CAX1 in Ca^{2+} homeostasis, CAX1 modifications could have effects on plant metabolism. Three *Brassica rapa* mutants for CAX1 were obtained through TILLING. The aim of this work is to assess the effect of the different mutations and different Ca^{2+} doses on plant metabolism. For this, the mutants and the parental line were grown under low, control and high Ca^{2+} doses and parameters related to nitrogen (N) and tricarboxylic acid (TCA) metabolisms, and amino acid (AAs) and phytohormone profiles were measured. The results show that *BraA.cax1a* mutations affect metabolism especially under high Ca^{2+} dose. Thus, *BraA.cax1a-7* inhibited some N metabolism enzymes and activated photorespiration activity. On the opposite side, *BraA.cax1a-12* mutation provides

a better tolerance to high Ca^{2+} dose. This tolerance could be provided by an improved N and TCA metabolisms enzymes, and a higher glutamate, malate, indole-3-acetic acid and abscisic acid concentrations. Therefore, *BraA.cax1a-12* mutation could be used for *B. rapa* improving; the metabolomics changes observed in this mutant could be responsible for a better tolerance to high Ca^{2+}.

Key words: Amino acids, CAX1, Nitrogen metabolism, Organic acids, Phytorhomones, Tricarboxylic acid cycle.

1. Introduction

One of the main mechanisms that plants possess to regulate internal Ca^{2+} homeostasis is through plant cation/H^+ exchangers (CAXs). CAXs transporters are crucial to generate Ca^{2+} profiles involved in signalling processes mediated by this cation (Pittman and Hirschi, 2016). Thus, Ca^{2+} alone or bound to calmodulin function as a secondary messenger that activates numerous signalling cascades that regulate diverse downstream targets leading to important physiological responses. There is 11 CAXs transporters described in *Brassica oleracea*. However, CAX1 is the most interesting because its expression not depend on the developmental stage and is highly correlated with Ca^{2+} accumulation (Lee *et al.*, 2013). In *Brassica rapa* two CAXs transporters (BraA.CAX1 and BraA.CAX3) are specially related with Ca^{+2} although BraA.CAX1 show a higher Ca^{+2} response and thereby its modification could have stronger effects on Ca^{2+} (Graham *et al.*, 2014).

Two very important pathways affected by Ca^{2+} are nitrogen (N) metabolism and tricarboxylic acid (TCA) cycle (White and Broadley, 2003). A correct regulation of N metabolism is basic given that is a major determinant of crop productivity and N is the basis of amino acids (AAs), proteins and nucleic acids (Kim *et al*., 2011). Several researches showed that Ca^{2+} availability and homeostasis can affect N metabolism. Thus, deficient or toxic Ca^{2+} applications cause the inhibition of N metabolism enzymes (Gao *et al*., 2011; Chao *et al*., 2008). Ca^{2+} homeostasis is as important as its levels, thus the application of compounds such alginate oligosaccharides enhance N metabolism and total N concentration by improving Ca^{2+} flux to the cytosol (Zhang *et al*., 2013). In addition, close related to N metabolism, photorespiration is important to maintain adequate N homeostasis and produce metabolites for other processes. However, the role of Ca^{2+} homeostasis in this process has not been well studied.

The primary assimilates of N are AAs such as glutamate (Glu), glutamine and aspartic acid (Asp). These and other AAs are efficiently catabolized by TCA cycle enzymes to produce energy for plant growth, especially in response to stress. Thus, N metabolism connects with respiration through TCA cycle (Galili *et al*., 2016). Likewise, TCA cycle plays a key role in plant physiology, being crucial in energy metabolism, providing AAs precursors, and being close related to other important processes such as photorespiration and ion balance (Igamberdiev and Eprintsev, 2016). Derived from TCA cycle an important anaplerotic pathway is carried out by phosphoenolpyruvate carboxylase (PEPC) that is able to replenish oxaloacetate. In addition, PEPC enzyme can fix CO_2 from

photorespiration and acts as a linking factor between C and N metabolism since the oxaloacetate produced is a precursor of malate, citrate and Asp (Sweetlove *et al*., 2010). Thus, citrate, malate and oxalate are three organic acids (OAs) that are both TCA cycle intermediaries and involved in the tolerance to metal toxicity, solubilisation of nutrients in the soil under deficiency conditions, their transport through the xylem and their vacuolar sequestration (Evangelou *et al*., 2007). TCA cycle is a highly regulated process, and one of the most important ways of regulation is through Ca^{2+} fluxes (Sweetlove *et al*., 2010).

Besides Ca^{2+}, phytohormones are another of the fundamental elements that act together with it in the regulation of plant metabolism. Phytohormones are compounds regulating general plant metabolism, highlighting the effect of cytokinins (CKs) on N metabolism (Sakakibara *et al*., 2006). Likewise, these compounds are involved in growth-control, development, response to stress and senescence. In addition, phytohormones induce changes on plant metabolism in order to improve nutrient uptake and homeostasis within the plant, and in turn, nutrients such as Ca^{2+} affect phytohormone synthesis and action (Marschner, 2012). On the other hand, Ca^{2+} is able to activate hormone synthesis and acts in signalling cascades in response to phytorhomones. In this way, Ca^{2+} is involved in abscisic acid (ABA), gibberellins (GAs), indole-3-acetic acid IAA and ethylene signalling through protein kinases that present a Ca^{2+} binding domain (Xu and Huang, 2017).

Given the crucial role of Ca^{2+} homeostasis in the regulation of plant metabolism and the role of CAX1 in Ca^{2+} homeostasis, CAX1 modifications could produce effects on plant metabolism. TILLING (Targeting Induced Local Lesions In Genomes) is a potential method to generate and identify CAX1 variants. This technique is a reverse-genetic tool in which seeds are treated with a chemical mutagen that produce mutations at high density, then mutants are screened to detect a certain mutation (Till, 2003). Three missense mutations were generated and identified through TILLING in the *B. rapa* ssp. trilocularis 'R-o-18' transporter *BraA.CAX1a*: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12* (Lochlainn *et al*., 2011). These mutations affect AAs upstream of the N-terminal autoinhibitory domain but that could change protein conformation and thereby affecting CAX1 function (Graham *et al*., 2014). Therefore, the objective of this work is to assess the effect of the different mutations in *BraA.cax1a* transporter on plant metabolism and to study its importance for a possible improvement of *B. rapa* and other related crop species.

2. Material and methods

2.1. Plant material, growth conditions, and treatments

Three *B. rapa* ssp. trilocularis 'R-o-18' mutants (*BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56) and the parent line R-o-18 were utilized as plant material for the experiment. Mutants plants were obtained and identified as described by Lochlainn *et al*. (2011) and Graham *et al*. (2014). Seeds were sown on filter paper moistened with milli-Q water (18.2 MV cm) in 9

cm Petri dishes. The dishes were incubated in the dark for 1 d at 4ºC before transferring to pots filled with vermiculite. These pots where placed in a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 22/18ºC (day/night) and 14/10-h photoperiod at a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Throughout the experiment the plants received a growth solution composed of 4 mM KNO_3, 3 mM NH_4NO_3, 2 mM $MgSO_4$ • 7 H_2O, 6 mM KH_2PO_4, 1 mM NaH_2PO_4 • 2 H_2O, 2 µM $MnCl_2$ • 4 H_2O, 1 µM ZnSO4·7H_2O, 0.25 µM $CuSO_4$ • 5 H_2O, 0.1 µM Na_2MoO_4 • 2 H_2O, 5 µM Fe-chelate (Sequestrene; 138FeG100), and 10 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was renewed every three days.

2.2. Experimental design and treatments

Treatments were started 30 days after germination and were kept for 21 days. Plants were grown with different Ca^{2+} doses: 0.4 mM of $CaCl_2$ as low Ca^{2+} dose, 4 mM of $CaCl_2$ as control Ca^{2+} dose, and 40 mM of $CaCl_2$ as high Ca^{2+} dose. During the experiments both Ca^{2+} dose applied (D) was varied on different mutants (M). The experimental design consisted of randomized complete block with 12 treatments, arranged in individual benches with eight plants per treatment and three replications each.

2.3. Plant sampling

Plant leaves were washed with distilled water, dried on filter paper, and weighed for fresh weight (FW). Half of the leaves from each treatment were

frozen at −30ºC for later biochemical assays and the other half of the plant material was lyophilized to measure the dry weight (DW) and the nutrients concentration.

2.4. Analysis of Ca^{2+} and N forms

For the Ca^{2+} concentration determination, a sample of 150 mg dry material was subjected to a mineralization process with sulfuric acid and H_2O_2 by the method of Wolf (1982) and then Ca^{2+} concentration was determined by ICP- MS.

NO_3^- was analysed from an aqueous extraction of 0.1 g of DW in 10 ml of Millipore-filtered water. A 100-µl aliquot was taken for NO_3^- determination and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO_3^- concentration by spectrophotometry (Cataldo *et al*., 1975). NH_4^+ was analysed from the aqueous extraction and total reduced N were obtained from digested samples and both were determined by the method described by Krom (1980).

2.5. N metabolism enzyme extractions and assays

Leaves were ground at 0ºC in 50 mM KH_2PO_4 buffer (pH 7.5) containing 2 mM EDTA, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 30,000 × *g* for 20 min. The resulting extract was used to measure enzyme activity of nitrate reductase (NR), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH). NR assay followed the methodology of Kaiser and Lewis (1984). GDH and GOGAT activities were assayed spectrophotometrically by

monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986).

For glyoxylate oxidase (GO) determination, fresh leaf tissue was ground with PVPP and 1 ml of 50 mM Tris–HCl buffer (pH 7.8) with 0.01% Triton X-100 and 5 mM DTT. The homogenate was centrifuged at 30,000 × *g* for 20 min. The supernatant was decanted and immediately used for the enzyme assay. GO activity was determined by following the formation of glyoxylate phenylhydrazone at 324 nm as described by Feierabend and Beevers (1972).

For determination of glutamate-glyoxylate aminotransferase (GGAT) activity, leaves were ground with 100 mM Tris–HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20,000 × *g* for 10 min. The resulting extract was used to measure enzyme activity. GGAT activity was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH (Igarashi *et al*., 2006).

Glutamine synthetase (GS) was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis (1984). Leaves were ground at 0ºC in 50 ml maleic acid-KOH buffer (pH 6.8) containing 100 mM sucrose, 2% (v/v) β-mercaptoethanol, and 20% (v/v) ethylene glycol. The homogenate was centrifuged at 30,000 × *g* for 20 min. The resulting extract was used to measure enzyme activity of GS. After incubation at 28ºC for 30 min, the

formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride.

Aspartate aminotransferase (AAT) activity was assayed spectrophotometrically at 340 nm using the method published by (Gonzalez *et al*., 1995). AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8), 4 mM $MgCl_2$, 10 mM aspartic acid, and enzyme extract.

The protein concentration of the extracts was determined according to the method of Bradford (1976) using bovine-serum albumin as the standard.

2.6. Soluble AAs analysis

Soluble AAs were extracted following the method of Bieleski and Turner (1966) with some modifications. 0.1 g of fresh leaves were homogenised in 1 ml of MCW (methanol: chloroform: water, 12:5:1). 50 µl of L-2 aminobutyric acid was added as an internal standard. The mixture was centrifuged at 2,300 × *g* for 10 min. To the resulting supernatant were added 700 µl of Milli-Q water and 1.2 ml of chloroform and incubated 24 h at 4 °C. Then, the aqueous phase was obtained, which was lyophilized and the resulting extract was diluted with 0.1 M HCl. Instrumental analysis of soluble AAs was carried out using the precolumn AccQ Tag Ultra Derivatization Kit (Waters, Milford, MA, USA). LC fluorescence analysis was performed on the Waters Acquity® UPLC System equipped with the Acquity

fluorescence detector. UPLC separation was performed on the AccQ Tag Ultra column (2.1 x 100 mm, 1.7 µm) from Waters. The flow rate was 0.7 mL min^{-1}, and the column temperature was kept at 55ºC. The injection volume was 1 µL, and the detection was set at a 266-nm excitation wavelength and a 473-nm emission wavelength. The solvent system consisted of two eluents: 1:20 Dilution of AccQ Tag Ultra eluent A concentrate and AccQ Tag Ultra eluent B.

2.7. Extraction and analysis of OAs

Malic, citric and oxalic acids were analysed according to (Gómez-Romero *et al.*, 2010) with some modifications. Briefly, 75 mg of freeze-dried and ground leaves were dropped in 1 ml of cold (-20°C) extraction mixture of methanol/water/acetic acid (80/19.5/0.5, v/v/v). Solids were separated by centrifugation (20,000 × *g*, 15 min) and re-extracted for 30 min at 4ºC in additional 1 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C_{18} cartridges (SepPak Plus, Waters, USA) and evaporated at 40ºC under vacuum to near dryness. The residue was dissolved in 1 ml water/methanol/acetic acid (94.5/5/0.5, v/v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA). 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The analytes were separated using a Zorbax SB-C18 HPLC column (5 µm, 150 x 0.5 mm, Agilent Technologies, Santa Clara, CA, USA), maintained at 30 ºC. Mass spectra were obtained using the Xcalibur software version 2.2

(ThermoFisher Scientific, Waltham, MA, USA). For OAs quantification, calibration curves were constructed for each analysed component (1, 2.5, 5, and 10 mg l^{-1}).

2.8. TCA enzyme extractions and assays

Extracts for measuring enzyme activities were obtained following the method of Li (2000), modified by grinding 0.1 g of leaves in 1 ml of extraction buffer containing 1 mM EDTA-Na, 10% glycerol,1% TritonX-100, 5 mM DTT and 1% polyvinylpyrrolidone (PVP) in 100 mM Tris–HCl pH 8.0. The slurry was centrifuged for 5 min at 20,000 × *g* and 4 ºC, and the supernatant was collected and analysed immediately. CS activity was assayed spectrophotometrically by monitoring the reduction of acetyl coenzyme A (CoA) to Co A with 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm (Srere, 1969). PEPC activity was measured using 2 mM phosphoenolpyruvate (PEP), 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.16 mM NADH and 100 mM of N,N-bis[2-hydroxyethyl]glycine (Bicine)-HCl, pH 8.5 (López-Millán *et al*., 2001). Finally MDH activity was determined with oxalate as substrate by measuring the decrease in absorbance at 340 nm due to the enzymatic oxidation of NADH (Dannel *et al*., 1995). The protein concentration of the extracts was determined using bovine-serum albumin as the standard Bradford (1976).

2.9. Hormone extraction and analysis

IAA, GAs (GA1, GA3 and GA4), CKs (tZ and iP), ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and ABA were analysed as in Ghanem *et al*., (2008) with some modifications. Briefly, 30 mg of homogenized

dry material were dropped in 0.5 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20,000 × *g*, 15 min) and re-extracted for 30 min at 4ºC in additional 0.5 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, USA) and evaporated at 40ºC under vacuum either to near dryness or until the organic solvent was removed. The residue was dissolved in 1 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through Millex nylon membrane filters 13 mm diameter of 0.22 µm pore size (Millipore, Bedford, MA, USA). Next, 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The mass spectra were determined using the Xcalibur software version 2.2. For quantification of plant hormones, calibration curves were constructed for each component analysed (1, 10, 50, and 100 µg l^{-1}) and corrected for 10 µg l^{-1} deuterated internal standards.

2.10. Statistical analysis

Data were subjected to a simple ANOVA at 95% confidence to evaluate the differences between treatments. A two-tailed ANOVA was applied to ascertain whether the Ca^{2+} doses, the mutations, or the interaction (D * M) significantly affected the results. Means were compared by Fisher's least significant differences (LSD). The significance levels for both analyses were expressed as * P<0.05, ** P<0.01, *** P<0.001, or NS (not significant). Principal Components Analysis (PCA) was utilized to assess relationships between treatments and all

parameters analysed. All statistical analyses were carried out employing the Statgraphics Centurion XVI software.

3. Results and discussion

3.1. Principal components analysis

Principal components analysis (PCA) was utilized to identify general trends in the data and to analyse the relationship between parameters. The first principal component (PC1) separated the samples with respect Ca^{2+} dose and accounted for 38.37% of the variance within the data. The second principal component (PC2) separated the samples into lines and accounted for 30.01% of the variance (Fig. 1). On the other hand, The PCA score scatter plot show that plants subjected to high Ca^{2+} dose are clearly separated from control and low doses. When plants were grown under low Ca^{2+}, *BraA.cax1a-12* separated from the other lines that did not show great differences between themselves. Under control Ca^{2+} dose, we observed that R-o-18 and *BraA.cax1a-12* are close related while *BraA.cax1a-7* separated clearly from the other lines. Under high Ca^{2+} dose lines were well separated (Fig. 1A).

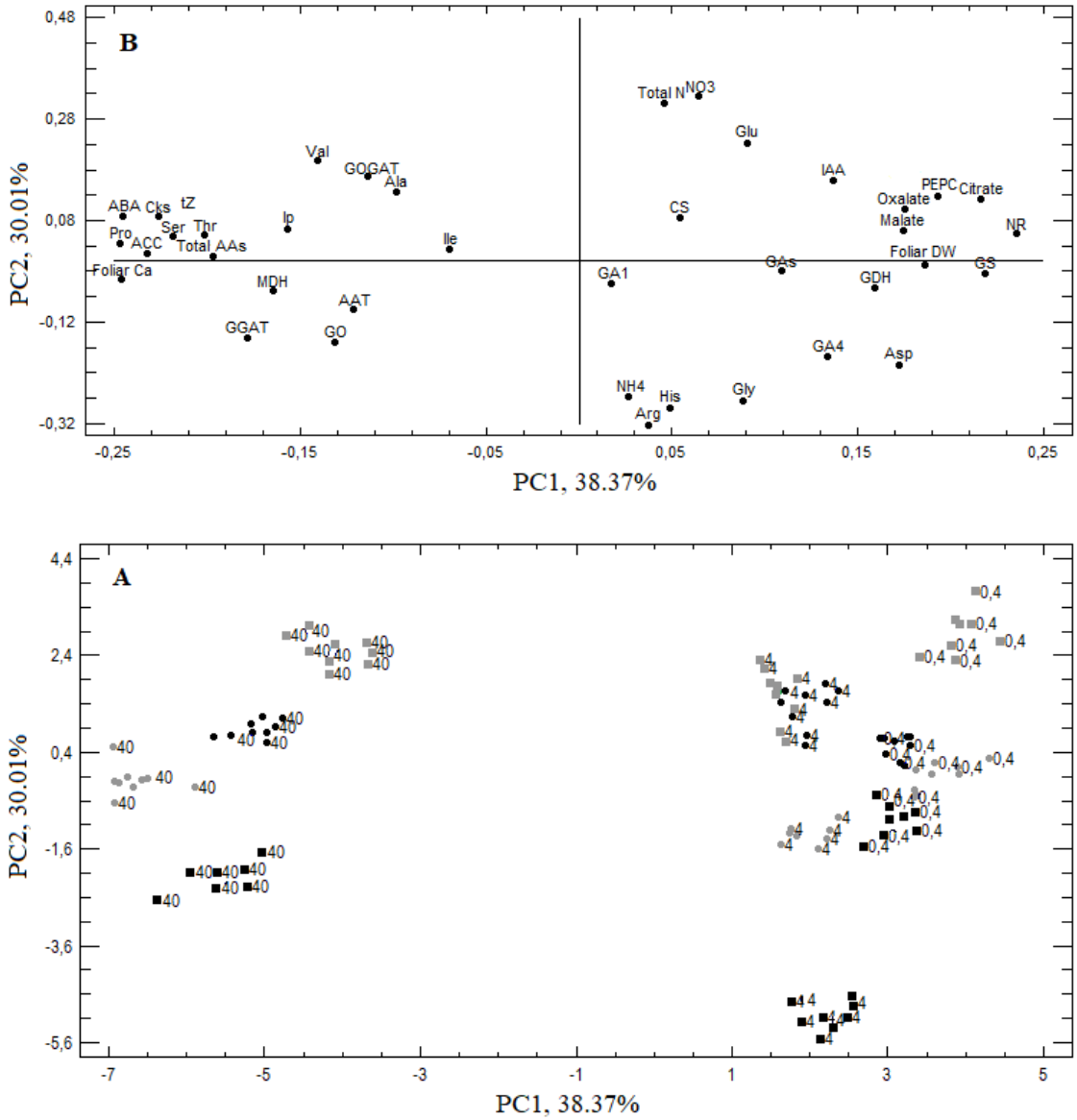

Fig. 1. Scores (A) and corresponding loadings plot (B) of principal component analysis (PCA) on all parameters analysed in R-o-18 (black dot), *BraA.cax1a-4* (grey dot), *BraA.cax1a-7* (black square), and *BraA.cax1a-12* (grey square) plants grown at three different Ca^{2+} doses (0.4, 4 and 40 mM)

3.2. Biomass and Ca^{2+} concentration

Several authors evidenced that modifications in CAX1 activity affect Ca^{2+} homeostasis and produce effects in plant physiology. Thus, *A. thaliana cax1* knockout mutants showed an altered growth and a decrease in Ca^{2+}

concentration especially when were grown under high Ca^{2+} dose (Catalá *et al*., 2003; Conn *et al*., 2011). On the other hand, plants with higher CAX1 activity accumulated more Ca^{2+} and present or not an altered phenotype, depending on the CAX1 modification and the species (Pittman and Hirschi, 2016). The *BraA.cax1a* mutants used in the present experiment did not present great alterations in their growth (Fig. 2). Indeed, plants grown under low and control Ca^{2+} doses did not present significant differences in leaf DW between mutants and R-o-18 (Fig. 2A). High Ca^{2+} dose caused a great reduction in foliar biomass in comparison to control Ca^{2+} dose (Fig. 2A). However, *BraA.cax1a-12* showed a lower foliar biomass loss and registered 82% more biomass than R-o-18 plants. Therefore, *BraA.cax1a-12* mutants could present some mechanism to avoid the Ca^{2+} toxicity observed in the rest of lines.

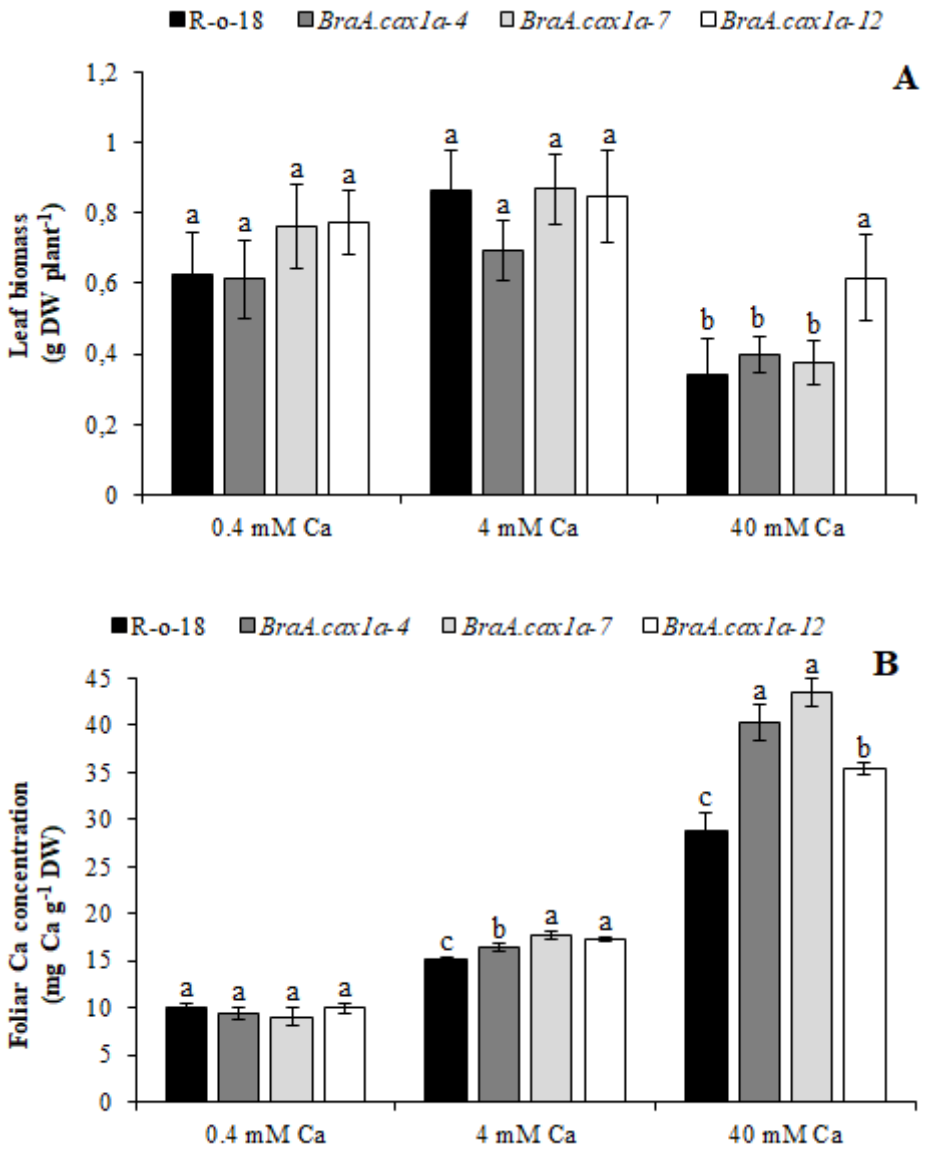

Fig. 2. Leaf biomass (A) and Ca^{2+} concentration (B) in *BraA.cax1a* mutants and R-o-18 plants grown under three Ca^{2+} doses. Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

BraA.cax1a-12 and R-o-18 presented a similar phenotype which is indicative that *BraA.cax1a-12* did not produce important alterations in plant physiology as previously reported by Navarro-León *et al*. (2018) and in the present work. In contrast, as reported by Graham *et al*. (2014), *BraA.cax1a-4* and

BraA.cax1a-7 mutants presented paler yellow leaves which is indicative of a higher stress and this could be associated to an altered metabolism as discussed below. These phenotypes are maintained regardless the Ca^{2+} dose applied.

Concerning foliar Ca^{2+} accumulation, when low Ca^{2+} was applied no significant differences were observed between lines. Nevertheless, as Ca^{2+} supply increased, the differences between lines in their foliar Ca^{2+} concentrations were more remarkable (Fig. 2B). Thus, *BraA.cax1a* mutations improved Ca^{2+} accumulation especially under high Ca^{2+} dose supply. However, this accumulation was lower in *BraA.cax1a-12* plants (Fig. 2B) that could store more efficiently Ca^{2+} in vacuoles as it was observed in other experiment in which a higher CAX1 activity enhance Ca^{2+} storage in this organelle (Pittman and Hirschi, 2016). This could provide *BraA.cax1a-12* an improved tolerance to Ca^{2+} toxicity.

3.3. N metabolism and photorespiration

It is well known that a proper Ca^{2+} supply and Ca^{2+} internal homeostasis is crucial for an optimal N metabolism performance (Gao *et al*., 2011; Chao *et al*., 2008; Zhang *et al*., 2013), and consequently *BraA.cax1a* mutations could altered N metabolism. In general, our results showed that both low and high Ca^{2+} applications reduced NR in all lines dose and GS activity was reduced by high Ca^{2+} dose in comparison with control Ca^{2+}. Regarding GDH, GS, and GOGAT enzymes, in general, we only observe differences between R-o-18 and mutants under low and high Ca^{2+} doses (Table 1; Fig. 3A). This suggest that *BraA.cax1a m*utations only affect to NH_4^+ assimilation process when non-optimal levels of

Ca^{2+} are applied to plants. Regarding photorespiration enzymes, GO and GGAT activities where higher when high Ca^{2+} dose was applied to the plants in comparison to control and low Ca^{2+} doses (Fig. 3B).

Table 1. N forms and NR activity in *BraA.cax1a* mutants and R-o-18 plants grown under three Ca^{2+} doses

		NO_3^- (mg g^{-1} DW)	NR (µM NO_2 mg $prot^{-1}$ min^{-1})	NH_4^+ (mg g^{-1} DW)	Total Reduced N (mg g^{-1} DW)
0.4 mM	R-o-18	19.36b	1.97a	3.21c	43.28a
	BraA.cax1a-4	29.92b	1.85a	3.36c	46.24a
	BraA.cax1a-7	27.01b	1.93a	4.33a	51.00a
	BraA.cax1a-12	84.80a	1.94a	3.84b	47.52a
	p-value	***	NS	***	NS
	$LSD_{0.05}$	24.92	0.24	0.26	9.17
4 mM	R-o-18	77.75a	3.09a	3.45b	42.60b
	BraA.cax1a-4	34.33b	2.80b	4.03a	46.30b
	BraA.cax1a-7	30.50b	2.00c	4.25a	61.01a
	BraA.cax1a-12	101.27a	2.98a	3.51b	60.03a
	p-value	***	***	***	***
	$LSD_{0.05}$	25.38	0.12	0.26	10.00
40 mM	R-o-18	24.27b	0.40b	3.70b	59.95b
	BraA.cax1a-4	10.47c	0.40b	3.54bc	35.13c
	BraA.cax1a-7	7.39c	0.30c	3.88a	29.56c
	BraA.cax1a-12	66.68a	0.88a	3.47c	78.72a
	p-value	***	***	**	***
	$LSD_{0.05}$	10.19	0.07	0.17	12.36
Analysis of variance					
Doses (D)		***	***	***	***
Mutation (M)		***	***	*	NS
D x M		***	***	***	***
$LSD_{0.05}$		7.15	0.09	0.15	5.96

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

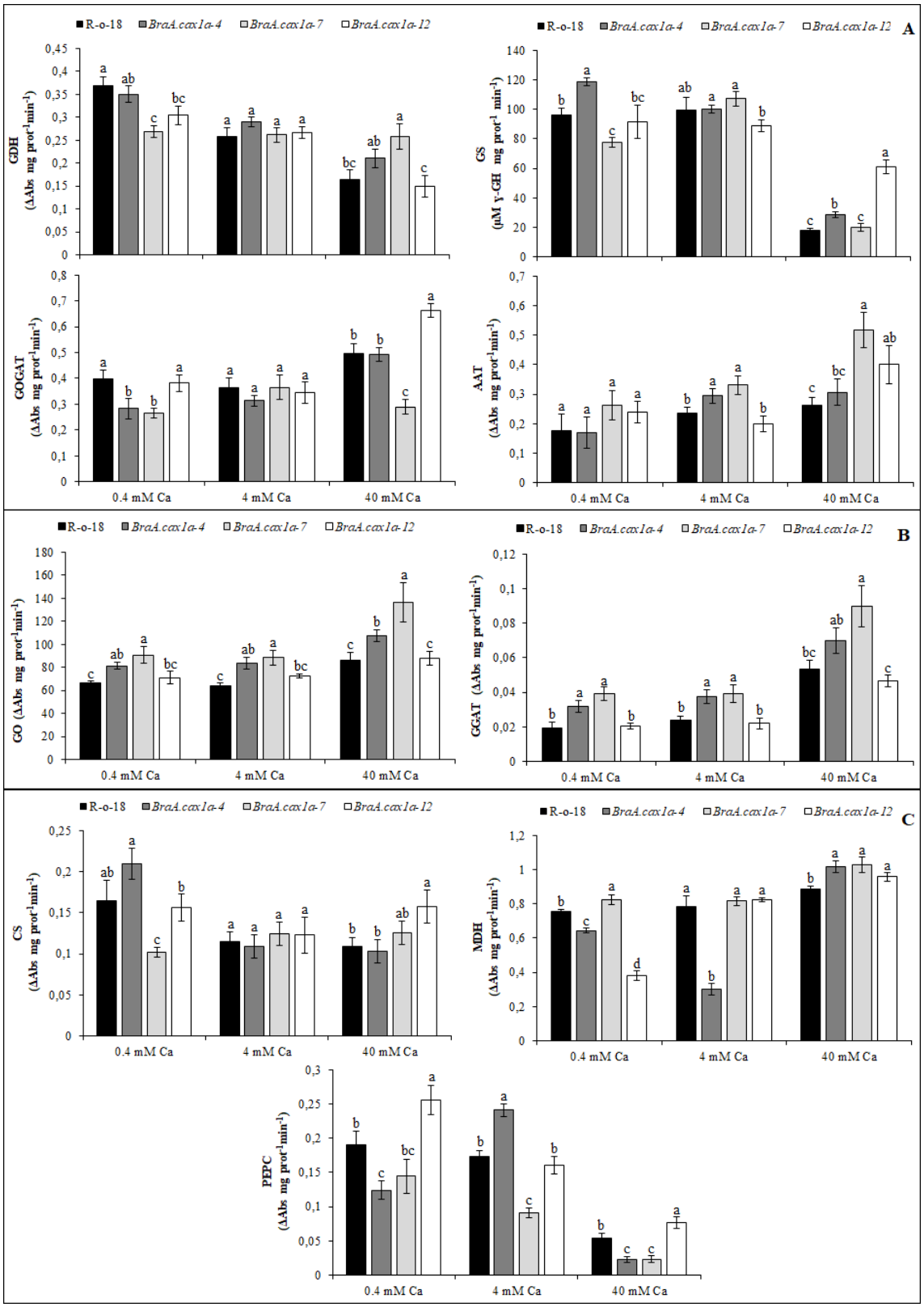

Fig. 3. Effect of *BraA.cax1a* mutations and Ca^{2+} doses on enzyme activities of N assimilation (A), photorespiration (B) and TCA cycle (C). Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

Comparing between lines, *BraA.cax1a-4* and specially *BraA.cax1a-7* reduced NO_3^- accumulation and GS/GOGAT cycle enzyme activities and increased NH_4^+ concentration and photorespiration enzymes with respect R-o-18 plants (Table 1; Fig. 3A-B). Under control Ca^{2+} dose, *BraA.cax1a-4* and *BraA.cax1a-7* presented lower NO_3^-, NR and higher NH_4^+ and AAT levels, while total reduced N was lower in *BraA.cax1a-4* in comparison to R-o-18 plants. High Ca^{2+} application decreased NO_3^- and total reduced N concentrations in *BraA.cax1a-4* plants while in *BraA.cax1a-7* NR activity was reduced (Table 1; Fig. 3A). The inhibition of N metabolism enzymes could be the cause of the lower total reduced N concentration in *BraA.cax1a-7* under higher Ca^{2+} dose (Table 1; Fig. 3A). In contrast, *BraA.cax1a-12* plants did not present an altered N metabolism under low and control Ca^{2+} doses and enhanced it under high Ca^{2+} dose in comparison to the other lines. Indeed, *BraA.cax1a-12* plants presented greater NO_3^- and total reduced N concentrations, lower NH_4^+ levels and higher NR, GS, GOGAT and AAT activities in comparison to R-o-18 plants (Table 1; Fig. 3A). These results could explain the higher biomass observed in *BraA.cax1a-12* under high Ca^{2+} because as observed by Kim *et al*. (2011) NO_3^- concentration and N metabolism is highly correlated with foliar DW.

According our results, *BraA.cax1a-7* mutant could have an enhanced GDH activity for a fast NH_4^+ assimilation preventing its toxicity under high Ca^{2+} supply. In turn, a greater GDH activity could contribute to a greater Glu concentration in this mutant. In addition, GDH enzyme could play this role under high Ca^{2+} conditions since a higher GDH activity in *BraA.cax1a-4* and *BraA.cax1a-7* mutants (Fig. 3A) could assimilate the excess of NH_4^+ produced by

photorespiration. Conversely, the lower GDH in *BraA.cax1a-7* and *BraA.cax1a-12* under low Ca^{2+} supply (Fig. 3A) could explain why NH_4^+ is accumulated in these plants (Table 1). These results support the role of Ca^{2+} in GDH activity to detoxify NH_4^+ excess. On the other hand, it was observed that photorespiration can be an important source of NH_4^+ and even the amount of NH_4^+ produced can overcome NH_4^+ produced by NR activity (Wingler *et al*., 2000). Therefore, the enhacenment in phtorespiration could cause the increment in NH_4^+ concentration (Table 1). Comparing between lines, *BraA.cax1a-4* and *BraA.cax1a-7* plants registered higher values of GO and GGAT activities while *BraA.cax1a-12* presented similar values in comparison to R-o-18. The highest GO and GGAT activities were found in *BraA.cax1a-7* plants grown under high Ca^{2+} dose (Fig. 3B). Therefore, the different *BraA.cax1a* mutations might affect these Ca^{2+} fluxes differently and consequently the activity of photorespiratory enzymes. A higher photorespiration rate usually is an adaptive response of plants suffering some kind of stress (Zhang and Peng, 2016). This agrees with previous results because *BraA.cax1a-4* and specially *BraA.cax1a-7* accumulated higher ROS concentrations which is indicative of a higher stress (Navarro-León *et al*., 2018).

3.4. Amino acid profile

PCA analysis related Ca^{2+} concentration to total AAs, and specifically Pro, Ser and Thr (Fig. 1B) because plants with high Ca^{2+} levels presented high levels of these AAs (Table 2). A decrease in growth as we observed under high Ca^{2+} dose usually causes an accumulation of AAs in the cytoplasm due to a decrease in the protein synthesis/degradation ratio (Atilio and Causin, 1996). Stressed plants present a higher proteolysis rate and accumulate certain AAs that protect

against stress such as Pro (Ashraf and Foolad, 2007). On the other hand, Gao *et al*. (2011) observed that Ca^{2+} deficiency reduced free AAs. This reduction also occurs in R-o-18 plants that presented lower AAs concentration under low Ca^{2+} but not in mutant lines (Table 2), so mutations could maintain AAs levels under Ca^{2+} deprivation. Under control Ca^{2+} dose, no differences in total AAs concentration were observed between lines. However, all mutants registered higher glycine (Gly) levels. In contrast with the majority of AAs, *BraA.cax1a* mutations reduced Val and Ile levels under control and high Ca^{2+} doses (Table 2). This reduction could be related to the fact that the synthesis and the catabolism of these AAs is close related. For intstance, branched-chain aminotransferases degradate these AAs in the mitochondria and could be activated as a consquence of *BraA.cax1a* mutations (Schuster and Binder, 2005).

Table 2. Amino acids concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca^{2+} doses

		Total AAs	Pro	Thr	Ser	Val	Ile	His	Arg
0.4 mM	R-o-18	33.23c	2.32b	2.77c	5.54b	0.53c	0.09c	0.89c	9.0
	BraA.cax1a-4	55.01b	1.39c	5.78ab	12.21a	0.73b	0.22b	2.34bc	14.
	BraA.cax1a-7	75.88ª	1.67c	4.57b	8.53ab	0.93a	0.31a	7.76a	32.
	BraA.cax1a-12	59.55b	4.00a	6.17a	12.09a	0.82ab	0.13c	2.99b	14.
	p-value	***	***	**	*	*	**	**	***
	$LSD_{0.05}$	7.60	0.45	1.41	3.84	0.17	0.07	1.68	1.7
4 mM	R-o-18	51.42ª	0.48d	4.47c	10.87a	1.01a	0.51a	1.43c	12.
	BraA.cax1a-4	57.17ª	6.88a	6.82ª	12.37a	0.50c	0.16b	1.59bc	10.
	BraA.cax1a-7	49.37ª	1.12c	5.11b	12.57a	0.59c	0.19b	2.14b	10.
	BraA.cax1a-12	54.77ª	3.66b	6.68a	11.95a	0.79b	0.12b	2.92a	12.
	p-value	NS	**	*	NS	*	***	**	NS
	$LSD_{0.05}$	17.07	0.62	0.60	4.14	0.17	0.09	0.63	0.7
40 mM	R-o-18	96.92ª	34.06a	9.01a	20.84ab	1.54a	0.42a	2.08a	14.
	BraA.cax1a-4	95.92ab	32.13a	10.12a	22.53a	0.90bc	0.30b	1.99a	12.
	BraA.cax1a-7	81.17b	27.09ab	7.19b	18.16bc	0.76c	0.19c	1.71ab	10.
	BraA.cax1a-12	70.67c	21.53b	7.05b	16.88c	1.09b	0.17c	1.50b	9.1
	p-value	*	*	*	*	**	*	*	*
	$LSD_{0.05}$	10.27	7.43	1.78	3.19	0.31	0.10	0.40	3.5
Analysis of variance									
Doses (D)		***	***	***	***	***	**	***	***
Mutation (M)		*	**	***	**	***	***	***	***
D x M		***	***	***	**	***	***	***	***
$LSD_{0.05}$		6.66	2.01	0.63	1.69	0.10	0.04	0.48	2.0

Values are expressed as μmol g^{-1} FW and differences between means(n=9) were compared by Fisher's least-signif
different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significa
(***)

PCA analysis also related NH_4^+ concentration with Arg and His levels (Fig. 1B). For these three parameters we observed a great increase in *BraA.cax1a-7* under low Ca^{2+} dose and a decrease in *BraA.cax1a-12* under high Ca^{2+} dose (Table 2). Arg and His are two AAs with high N content and are mainly used as N storage. Likewise, Asp is one of the main free AAs in plant for N transport within the plant. AAT produce a bulk of Asp that serves as a N reserve (Galili *et al*., 2016). In this way, the increase in Asp levels in *BraA.cax1a-4* and *BraA.cax1a*-7 plants (Table 2) is also supported by a higher AAT activity (Fig. 3A). Therefore, *BraA.cax1a-7* probably presents a higher N remobilization through these AAs and in *BraA.cax1a-12* is reduced due to the plant is suffering less stress and N remobilization could not be necessary. On the other hand, *BraA.cax1a-12* registered higher Glu than the other lines (Table 2). Despite the general decrease in AAs, Glu did not decrease and probably is due to its importance in N metabolism (Galili *et al*., 2016). PCA showed a close relation between Glu, NO_3^- and total reduced N (Fig. 1B) and this could be due to Glu is one of the most concentrated AAs and its synthesis is directly related with GS/GOGAT enzymes that are activated by NO_3^- levels. Thus, *BraA.cax1a-12* mutant presented the greatest levels for these parameters (Table 1; Fig. 3A). On the other hand, stress usually cause Pro and Thr accumulations. These AAs, especially Pro, are considered attenuation agents for abiotic stress playing a role in osmotic adjustment, ROS elimination, and stabilization of subcelluar structures (Ashraf and Foolad, 2007). All plants in our experiment showed a clear increase in Pro and Thr accumulations caused by Ca^{2+} toxicity. However this increase was much lower in *BraA.cax1a-12* plants which indicates lower stress in this mutant (Table 2).

3.5. OAs concentration and TCA cycle enzyme activities

Plants synthetize and accumulated OAs such as malate, citrate and oxalate to increase nutrient uptake under deficiency conditions or to improve homeostasis under toxicity conditions (Evangelou *et al*., 2007). In the present experiment, all lines showed higher OAs concentrations under low Ca^{2+} dose in comparison to control Ca^{2+} dose, probably in order to increase Ca^{2+} uptake. However, under high Ca^{2+} dose, OAs did not appear to improve Ca^{2+} homeostasis since their concentrations decreased markedly, except malate in *BraA.cax1a-12* mutant (Fig. 4). Comparing between lines, low Ca^{2+} application unequally affected to citrate concentration depending on the *BraA.cax1a* mutation while all mutations increased malate (Fig. 4A and 4B). Malate could be crucial in the tolerance of high Ca^{2+} levels because it is the main OA that binds to Ca^{2+} in vacuoles. In addition malate has a positive influence on N metabolism (Igamberdiev and Eprintsev, 2016). This could be important in *BraA.cax1a-12* mutant to cope with Ca^{2+} toxicity. Likewise, this mutant maintained citrate levels in contrast with the other mutants in which citrate levels decreased under control and high Ca^{2+} doses (Fig. 4B). On the other hand, oxalate crystal formation has a function of Ca^{2+} storage in vacuoles, as well as the maintenance of concentration gradient between the vacuole and the cytosol (Igamberdiev and Eprintsev, 2016). This role of oxalate might be important in *BraA.cax1a-7* and *BraA.cax1a-12* plant grown under low Ca^{2+} dose as they presented higher levels than the other lines (Fig. 4C). In contrast, *BraA.cax1a-4* and *BraA.cax1a-7* presented very low concentration of oxalate under high Ca^{2+} dose (Fig. 4C) which suggest that these mutations may alter oxalate synthesis or degradation.

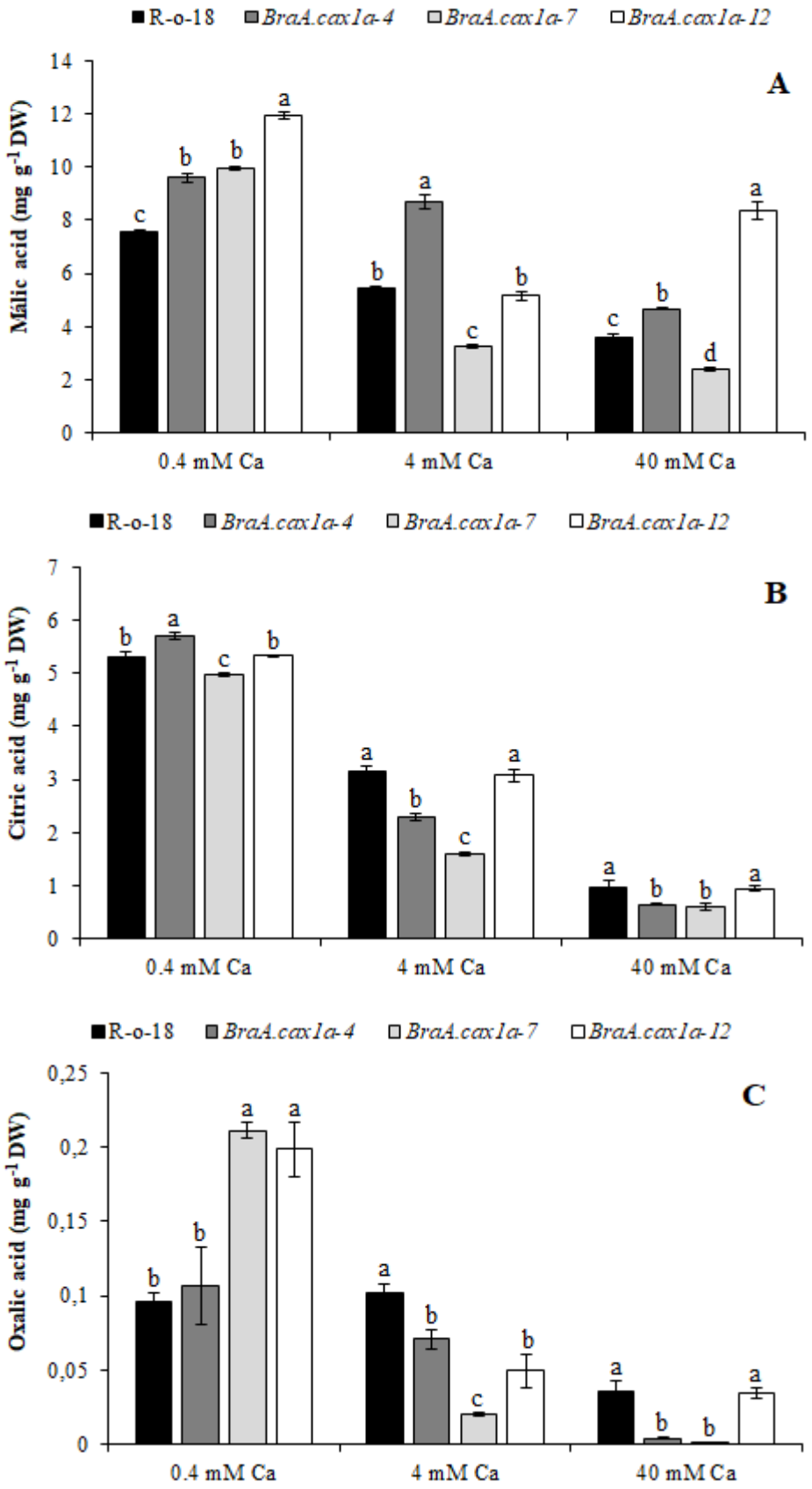

Fig. 4. Effect of *BraA.cax1a* mutation and Ca^{2+} doses on concentration of main organic acids. Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

Regarding TCA cycle, Ca^{2+} supply influence TCA cycle enzyme activities in *B. rapa* plant and other species (Blasco *et al*., 2015). In the present experiment, CS activity was not especially affected by *BraA.cax1a* mutations, only was reduced in *BraA.cax1a-7* plants grown under low Ca^{2+} dose. Conversely, all mutations increased MDH activity under high Ca^{2+} dose (Fig. 3C). *BraA.cax1a-12* mutation enhaced CS and PEPC activites under Ca^{2+} toxicity (Fig. 3C), which could contribute to maintain a higher biomass in this mutant. An increase in TCA activity may give plants tolerance against stress due to Ca^{2+} toxicity. More specifically, PEPC activity is involved in several physiological processes and acts as a linking factor between C and N metabolisms (Sweetlove *et al*., 2010). The greater PEPC activity in *BraA.cax1a-12* can be a positive response of tolerance to stress in both conditions of Ca^{2+} deficiency and especially of Ca^{2+} toxicity (Fig. 3C). PEPC activity connects with N metabolism as it produces OAA that is used by AAT enzyme that increased in *BraA.cax1a-12* plants (Fig. 3A). On the other hand, AAs are effciently catabolized by TCA cycle enzymes to produce cellular energy, especially under energy deprivation caused by stress (Galili *et al*., 2016). *BraA.cax1a-4* and *BraA.cax1a-7* might use the higher free AAs concentration (Table 2) to be catabolized in TCA cycle to produce energy.

3.6. Phytohormone profile

CAX1 mutations could affect both concentration and responses of phytorhomones in plants because Ca^{2+} is involved in phytohormone signalling and synthesis (Xu and Huang, 2017). In fact, Cheng *et al*. (2003) observed that IAA signalling is modified by CAX1 mutations in *Arabidopsis thaliana*. Likewise, when cax1 is knock out, plants loss sensibility to ethylene and ABA (Zhao *et al*.,

2008). In the present experiment, *BraA.cax1a* mutations affected to phytohormone profile, highlighting an increase in IAA levels regardless the Ca^{2+} dose applied (Table 3). IAA synthesis and accumulation could be close related to Ca^{2+} fluxes produced by CAX1 transporters. On the other hand, *BraA.cax1a-7* and *BraA.cax1a-12* increased phytohormone levels under low Ca^{2+} dose (Table 3). These two mutants presented high average DW (Fig. 2A), although not significant, this mutation could enhance tolerance to Ca^{2+} deficiency.

Table 3. Phytohormones concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca^{2+} do

		IAA	GA1	GA3	GA4	GAs	tZ	iP	CKs
0.4 mM	R-o-18	7.64d	0.04b	nd	0.02c	0.06b	171.28c	2.28b	173.
	BraA.cax1a-4	13.00c	0.05ab	nd	0.04b	0.10b	180.92bc	1.54c	182.
	BraA.cax1a-7	21.43b	0.07a	0.07	0.07a	0.21a	199.15b	3.09a	214.
	BraA.cax1a-12	33.29a	0.08a	0.16	0.02c	0.25a	244.35a	1.18c	245.
	p-value	***	NS		***	***	***	***	**
	$LSD_{0.05}$	3.95	0.03		0.01	0.05	21.16	0.66	23.5
4 mM	R-o-18	5.28c	0.22a	nd	0.03ab	0.25a	216.56a	2.07a	218.
	BraA.cax1a-4	8.91b	0.10bc	nd	0.05a	0.15b	244.201a	1.07b	245.
	BraA.cax1a-7	10.58b	0.17ab	0.04	0.04ab	0.25a	181.92b	1.30b	185.
	BraA.cax1a-12	16.63a	0.07c	0.05	0.03b	0.14b	215.47a	2.55a	218.
	p-value	***	**		NS	*	**	***	*
	$LSD_{0.05}$	3.62	0.08		0.02	0.09	29.07	0.56	29.8
40 mM	R-o-18	3.90d	0.13a	nd	0.02a	0.15a	246.02d	1.70c	247.
	BraA.cax1a-4	7.02b	0.07bc	nd	0.02a	0.09b	374.85a	4.72b	379.
	BraA.cax1a-7	5.31c	0.03c	0.03	0.02a	0.08b	335.25b	2.84bc	338.
	BraA.cax1a-12	11.33a	0.10ab	nd	0.02a	0.14a	291.08c	3.55ab	294.
	p-value	***	*		NS	**	***	*	***
	$LSD_{0.05}$	1.40	0.05		0.01	0.04	29.02	1.64	29.4
Analysis of variance									
Doses (D)		***	***		***	***	***	***	***
Mutation (M)		***	**		***	***	***	NS	***
D x M		***	***		***	***	***	***	***
$LSD_{0.05}$		1.72	0.03		0.01	0.03	13.78	0.55	14.2

Values are expressed as ng g^{-1} DW and differences between means (n=9) were compared by Fisher's least-significance
letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.

According PCA analysis, IAA levels are related to foliar DW (Fig. 1B). The lowest IAA levels are found under high Ca^{2+} dose as well as foliar DW. In addition, *BraA.cax1a-12* presented the highest IAA levels and also the highest biomass (Table 3; Fig. 2A). Therefore, this suggest that IAA could be key to maintain biomass under high Ca^{2+} conditions. On the other hand, under high Ca^{2+} dose CKs levels correlated well with Ca^{2+} accumulation. Thus, mutants accumulated more Ca^{2+} and presented higher CKs level and *BraA.cax1a-12* presented less CKs and less Ca^{2+} than the other mutant lines (Table 3; Fig. 2B), suggesting that high CKs levels could stimulate Ca^{2+} absorption. In addition, CKs were proved to act as a long-range messenger in N signalling and to activate genes encoding enzymes involved in N and C metabolisms (Sakakibara *et al*., 2006). In our study the stress produced by Ca^{2+} might induce CKs synthesis or accumulation in order to induce these genes. In similar way to CKs, GAs and specifically GA3 influence Ca^{2+} accumulation in some species (Yang *et al*., 2011). However, in the present experiment GAs concentration was not related with Ca^{2+} accumulation (Fig. 1B). *BraA.cax1a-12* was the unique line that maintained its GAs levels while in the rest of lines GAs levels decreased under high Ca^{2+} application with respect its control levels.

Regarding stress related hormones, such as ABA and ethylene, *BraA.cax1a-4* mutation presented the same levels than R-o-18 in contrast to the other mutants (Table 3). Therefore, not all *BraA.cax1a* mutations affected to phytohormone levels. ABA induces cytosolic Ca^{2+} elevation, which is in part due to Ca^{2+} release from the vacuole where CAX1 transporter is found. That is why ABA could modify cellular Ca^{2+} distribution and Ca^{2+} concentration in plants (De

Freitas *et al*., 2013). Likewise, ABA is a stress related hormone and is known to reduce plant growth. However, *BraA.cax1a-12* presented the highest ABA levels under high Ca^{2+} conditions (Table 3). The higher ABA levels could be compensated by the great increase in IAA concentration. In addition, *BraA.cax1a-12* presented lower ACC levels. Ethylene, gaseous hormone synthetized from ACC, increase its synthesis when plants are under stress. Likewise, ethylene influences nutrient uptake and it is involved in plant responses under growth-limiting conditions (Iqbal *et al*., 2013). In the present experiment foliar Ca^{2+} concentration may be related to ACC because this hormone increased with Ca^{2+} concentration (Fig. 1B). However, it can not be stated that ACC promotes Ca^{2+} accumulation because R-o-18 presented the higher ACC levels but the lower Ca^{2+} accumulation under high Ca^{2+} dose supply (Table 3; Fig. 2B).

4. Conclusions

The results obtained in this work show that *BraA.cax1a* mutations clearly affect plant metabolism. These effects depend on the mutation in CAX1a transporter and were more remarkable in plants grown under high Ca^{2+} dose. Thus, *BraA.cax1a-4* and specially *BraA.cax1a-7* mutations probably produce alterations in Ca^{2+} homeostasis that impaired some N metabolism enzymes and activated photorespiration activity. In addition, all mutations produce alterations in AAs accumulation. For instance, *BraA.cax1a-7* plants probably increase N remobilization AAs being all these results indicative of a greater stress. On the opposite side, *BraA.cax1a-12* mutation provides a better tolerance to high Ca^{2+} doses. This tolerance could be provided by improved N metabolism enzyme

activities, PEPC and CS activities, and by a higher Glu, malate, IAA and ABA concentrations. Therefore, *BraA.cax1a-12* mutation could be useful in Ca^{2+} biofortification programs employing *B. rapa* plants and the metabolomics changes observed in this mutant could be key for a greater tolerance to high Ca^{2+} doses.

Acknowledgments

We thank Dr. Martin R. Broadley and Dr. Neil Graham from Nottingham University for providing us the seeds utilized in this experiment.

Funding

This work was supported by the PAI programme (Plan Andaluz de Investigación, Grupo de Investigación AGR282) and by a Grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL [FPU14/01858]

References

Ashraf, M., Foolad, M.R., 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 59, 206–216. https://doi.org/10.1016/j.envexpbot.2005.12.006

Atilio, B., Causin, H.U.F., 1996. The central role of amino acids on nitrogen utilization and plant growth. J. Plant Physiol. 362, 358–362. https://doi.org/10.1016/S0176-1617(96)80134-9

Bieleski, R.L., Turner, N.A., 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Anal. Biochem. 17, 278–93. https://doi.org/https://doi.org/10.1016/0003-2697(66)90206-5

Blasco, B., Graham, N.S., Broadley, M.R., 2015. Antioxidant response and carboxylate metabolism in *Brassica rapa* exposed to different external Zn, Ca, and Mg supply. J. Plant Physiol. 176, 16–24. https://doi.org/10.1016/j.jplph.2014.07.029

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Catalá, R., Santos, E., Alonso, J.M., Ecker, J.R., Martínez-Zapater, J.M., Salinas, J., 2003. Mutations in the Ca^{2+}/H^{+} transporter CAX1 increase CBF/DREB1 expression and the cold-acclimation response in *Arabidopsis*. Plant Cell 15, 2940–2951. https://doi.org/10.1105/tpc.015248

Cataldo, D.A., Maroon, M., Schrader, L.E., Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6, 71–80. https://doi.org/10.1080/00103627509366547

Chao, L., Bofu, P., Weiqian, C., Yun, L., Hao, H., Liang, C., Xiaoqing, L., Xiao, W., Fashui, H., 2008. Influences of calcium deficiency and cerium on growth of spinach plants. Biol. Trace Elem. Res. 121, 266–275. https://doi.org/10.1007/s12011-007-8054-y

Cheng, N.H., Pittman, J.K., Barkla, B.J., Shigaki, T., Hirschi, K.D., 2003. The Arabidopsis *cax1* mutant exhibits impaired ion homeostasis, development, and hormonal responses and reveals interplay among vacuolar transporters. Plant Cell 15, 347–364. https://doi.org/10.1105/tpc.007385.348

Conn, S.J., Gilliham, M., Athman, A., Schreiber, A.W., Baumann, U., Moller, I., Cheng, N.-H., Stancombe, M.A., Hirschi, K.D., Webb, A.A.R., Burton, R., Kaiser, B.N., Tyerman, S.D., Leigh, R.A., 2011. Cell-specific vacuolar calcium storage mediated by CAX1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis*. Plant Cell 23, 240–257. https://doi.org/10.1105/tpc.109.072769

Dannel, F., Pfeffer, H., Marschner, H., 1995. Isolation of apoplasmic fluid from sunflower leaves and its use for studies on influence of nitrogen supply on apoplasmic pH. J. Plant Physiol. 146, 273–278. https://doi.org/10.1016/S0176-1617(11)82053-5

de Freitas, S.T., McElrone, A.J., Shackel, K.A., Mitcham, E.J., 2013. Calcium partitioning and allocation and blossom-end rot development in tomato plants in

response to whole-plant and fruit-specific abscisic acid treatments. J. Exp. Bot. 65, 235–247

Evangelou, M.W.H., Ebel, M., Schaeffer, A., 2007. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. Chemosphere 68, 989–1003. https://doi.org/10.1016/j.chemosphere.2007.01.062

Feierabend, J., Beevers, H., 1972. Developmental studies on microbodies in wheat leaves. Planta 123, 63–77. https://doi.org/10.1007/BF00388061

Galili, G., Amir, R., Fernie, A.R., 2016. The regulation of essential amino acid synthesis and accumulation in plants. Annu. Rev. Plant Biol. 67, 153–178. https://doi.org/10.1146/annurev-arplant-043015-112213

Gao, H., Jia, Y., Guo, S., Lv, G., Wang, T., Juan, L., 2011. Exogenous calcium affects nitrogen metabolism in root-zone hypoxia-stressed muskmelon roots and enhances short-term hypoxia tolerance. J. Plant Physiol. 168, 1217–1225. https://doi.org/10.1016/j.jplph.2011.01.022

Ghanem, M.E., Albacete, A., Martínez-Andújar, C., Acosta, M., Romero-Aranda, R., Dodd, I.C., Lutts, S., Pérez-Alfocea, F., 2008. Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.). J. Exp. Bot. 59, 3039–50. https://doi.org/10.1093/jxb/ern153

Gómez-Romero, M., Segura-Carretero, A., Fernández-Gutiérrez, A., 2010. Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. Phytochemistry 71, 1848–64. https://doi.org/10.1016/j.phytochem.2010.08.002

Gonzalez, E.M., Gordon, A.J., James, C.L., Arrese-Igor, C., 1995. The role of sucrose synthase in the response of soybean nodules to drought. J. Exp. Bot. 26, 1515–1523

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of *Brassica* under altered Ca supply identifies *Arabidopsis* Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Groat, R.G., Vance, C.P., 1981. Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.) Developmental patterns and response to applied nitrogen. Plant Physiol. 67, 1198–1203. https://doi.org/10.1104/pp.67.6.1198

Igamberdiev, A.U., Eprintsev, A.T., 2016. Organic acids: The pools of fixed carbon involved in redox regulation and energy balance in higher plants. Front. Plant Sci. 7, 1042. https://doi.org/10.3389/fpls.2016.01042

Igarashi, D., Tsuchida, H., Miyao, M., Ohsumi, C., 2006. Glutamate:glyoxylate aminotransferase modulates amino acid content during photorespiration. Plant Physiol. 142, 901–910. https://doi.org/10.1104/pp.106.085514

Iqbal, N., Trivellini, A., Masood, A., Ferrante, A., Khan, N.A., 2013. Current understanding on ethylene signaling in plants: the influence of nutrient availability. Plant Physiol. Biochem. 73, 128–38. https://doi.org/10.1016/j.plaphy.2013.09.011

Kaiser, J.J., Lewis, O.A.M., 1984. Nitrate reductase and glutamine synthetase activity in leaves and roots of nitrate-fed *Helianthus annuus* L. Plant Soil 77, 127–130. https://doi.org/10.1007/bf02182818

Kim, H.-Y., Lim, S.-S., Kwak, J.-H., Lee, D.-S., Lee, S.-M., Ro, H.-M., Choi, W.-J., 2011. Dry matter and nitrogen accumulation and partitioning in rice (*Oryza sativa* L.) exposed to experimental warming with elevated CO_2. Plant Soil 342, 59–71. https://doi.org/10.1007/s11104-010-0665-y

Krom, M.D., 1980. Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. Analyst 105, 305–316. https://doi.org/10.1039/an9800500305

Lee, J., Park, I., Lee, Z.-W., Kim, S.W., Baek, N., Park, H.-S., Park, S.U., Kwon, S., Kim, H., 2013. Regulation of the major vacuolar Ca^{2+} transporter genes, by intercellular Ca^{2+} concentration and abiotic stresses, in tip-burn resistant *Brassica oleracea*. Mol. Biol. Rep. 40, 177–188. https://doi.org/10.1007/s11033-012-2047-4

Li, X.F., 2000. Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. Plant Physiol. 123, 1537–1544. https://doi.org/10.1104/pp.123.4.1537

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R.,

2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

López-Millán, A.F., Morales, F., Abadía, A., Abadía, J., 2001. Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (*Beta vulgaris*) leaves. Physiol. Plant. 112, 31–38. https://doi.org/10.1034/j.1399-3054.2001.1120105.x

Marschner, H., 2012. Mineral Nutrition of Higher Plants. London

Navarro-León, E., Ruiz, J.M., Graham, N., Blasco, B., 2018. Physiological profile of CAX1a TILLING mutants of *Brassica rapa* exposed to different calcium doses. Plant Sci. 272, 164–172. https://doi.org/10.1016/j.plantsci.2018.04.019

Obata, T., Fernie, A.R., 2012. The use of metabolomics to dissect plant responses to abiotic stresses. Cell. Mol. Life Sci. 69, 3225–3243. https://doi.org/10.1007/s00018-012-1091-5

Pittman, J.K., Hirschi, K.D., 2016. CAX-ing a wide net: Cation/H^+ transporters in metal remediation and abiotic stress signaling. Plant Biol. 18, 741–749. https://doi.org/10.1111/plb.12460

Sakakibara, H., Takei, K., Hirose, N., 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends Plant Sci. 11, 440–448. https://doi.org/10.1016/j.tplants.2006.07.004

Schuster, J., Binder, S., 2005. The mitochondrial branched-chain aminotransferase (AtBCAT-1) is capable to initiate degradation of leucine, isoleucine and valine in almost all tissues in *Arabidopsis thaliana*. Plant Mol. Biol. 57, 241–254. https://doi.org/10.1007/s11103-004-7533-1

Singh, R.P., Srivastava, H.S., 1986. Increase in glutamate synthase (NADH) activity in maize seedlings in response to nitrate and ammonium nitrogen. Physiol. Plant. 66, 413–416. https://doi.org/10.1111/j.1399-3054.1986.tb05944.x

Srere, P.A., 1969. Citric acid cycle, Methods in Enzymology, Methods in Enzymology. Elsevier. https://doi.org/10.1016/0076-6879(69)13005-0

Sweetlove, L.J., Beard, K.F.M., Nunes-Nesi, A., Fernie, A.R., Ratcliffe, R.G., 2010. Not just a circle: flux modes in the plant TCA cycle. Trends Plant Sci. 15, 462–470. https://doi.org/10.1016/j.tplants.2010.05.006

Till, B.J., 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res. 13, 524–530. https://doi.org/10.1101/gr.977903

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511. https://doi.org/10.1093/aob/mcg164

Wingler, A., Lea, P.J., Quick, W.P., Leegood, R.C., 2000. Photorespiration: metabolic pathways and their role in stress protection. Philos. Trans. R. Soc. London. Ser. B Biol. Sci. 355, 1517–1529. https://doi.org/10.1098/rstb.2000.0712

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Xu, W., Huang, W., 2017. Calcium-dependent protein kinases in phytohormone signaling pathways. Int. J. Mol. Sci. 18, 2436. https://doi.org/10.3390/ijms18112436

Xu, Z., Lei, P., Feng, X., Xu, X., Liang, J., Chi, B., 2014. Calcium involved in the poly (γ-glutamic acid)-mediated promotion of Chinese cabbage nitrogen metabolism. Plant Physiol. Biochem. 80, 144-152. https://doi.org/10.1016/j.plaphy.2014.03.036

Yang, H., Jie, Y., Shao, X., 2011. Regulation of calcium uptake and translocation in plants. Symp. Integr. Canopy, Rootstock Environ. Physiol. Orchard Syst. 1011–1016

Zhang, Y.H., Yin, H., Liu, H., Wang, W.X., Wu, L.S., Zhao, X.M., Du, Y.G., 2013. Alginate oligosaccharides regulate nitrogen metabolism via calcium in *Brassica campestris* L. var. *utilis* Tsen et Lee. J. Hortic. Sci. Biotechnol. 88, 502–508. https://doi.org/10.1080/14620316.2013.11512998

Zhang, Z.S., Peng, X.X., 2016. Multifunctional roles of photorespiration and its regulation for the balance. https://doi.org/10.13592/j.cnki.ppj.2016.1017

Zhao, J., Barkla, B.J., Marshall, J., Pittman, J.K., Hirschi, K.D., 2008. The *Arabidopsis cax3* mutants display altered salt tolerance, pH sensitivity and reduced plasma membrane H^+-ATPase activity. Planta 227, 659–669. https://doi.org/10.1007/s00425-007-0648-2

CAPÍTULO 2: TOLERANCIA A LA TOXICIDAD DE CADMIO Y EL POTENCIAL PARA SU FITORREMEDIACIÓN DE TRES MUTANTES CAX1A TILLING DE *Brassica rapa*

Resumen

El cadmio (Cd) es uno de los metales pesados más tóxicos que reduce la productividad de los cultivos y es una amenaza para toda la cadena alimentaria, incluida la salud humana. La fitorremediación es una estrategia respetuosa con el medio ambiente para limpiar el suelo contaminado con metales pesados. Los investigadores están seleccionando nuevas variedades con una mayor capacidad para fines de fitorremediación. Tres mutantes de *Brassica rapa* para el transportador CAX1 se obtuvieron a través de TILLING. El objetivo de este trabajo es evaluar la tolerancia de estos mutantes a la toxicidad del Cd y su potencial para la fitorremediación del Cd. Para ello, se cultivaron los mutantes y el parental R-o-18 bajo condiciones control y de toxicidad del Cd (100 µM $CdCl_2$) y se analizaron parámetros fisiológicos, de crecimiento y de acumulación de Cd. Los resultados muestran que la mutación *BraA.cax1a* proporciona una mayor capacidad de absorción de Cd, aunque sólo *BraA.cax1a-12* sería útil para la fitorremediación porque registró más del triple del contenido de Cd de R-o-18 y presentó una mejor tolerancia al Cd. Esta tolerancia podría deberse a las acumulaciones más elevadas de Ca y Mg, al mantenimiento del rendimiento de la fotosíntesis, a la may7or desintoxicación de ROS y de actividad de los ciclos

de AsA/GSH y TCA, a las mayores concentraciones de malato y GA4 y a los menores niveles de etileno. Este estudio identifica a *BraA.cax1a-12* como un potencial mutante para la fitorremediación del suelo contaminado con Cd e identifica posibles elementos fisiológicos que contribuyen a esta capacidad.

Adapted from Ecotoxicology and environmental safety, 189, 109961.

https://doi.org/10.1016/j.ecoenv.2019.109961

Tolerance to cadmium toxicity and phytoremediation potential of three *Brassica rapa* CAX1a TILLING mutants

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Abstract

Cadmium (Cd) is one of the most toxic heavy metals that reduces crop productivity and is a threat to all the food chain including human health. Phytoremediation is an environmentally friendly strategy to clean up soil contaminated with heavy metals. Researchers are selecting new varieties with an enhanced capacity for phytoremediation purposes. Three *Brassica rapa* mutants for CAX1 transporter were obtained through TILLING. The objective of this work is to evaluate the tolerance of these mutants to Cd toxicity and its potential for Cd phytoremediation. For this, the mutants and the parental R-o-18 were grown under control and Cd toxicity conditions (100 µM $CdCl_2$) and growth, Cd accumulation and physiological parameters were analyzed. The results show

that *BraA.cax1a* mutation provides greater Cd uptake capacity although only *BraA.cax1a-12* would be useful for phytoremediation because it registered more than three-fold the Cd content of R-o-18 and presented greater Cd tolerance. This tolerance could be due to the higher Ca and Mg accumulations, the maintaining of photosynthesis performance, the enhanced ROS detoxification and AsA/GSH and TCA cycles, the higher malate, and GA4 concentrations and the lower ethylene levels. Briefly, this study identifies *BraA.cax1a-12* as a potential mutant for phytoremediation of Cd contaminated soil and identifies possible physiological elements that contribute to this capacity.

Keywords**:** *Brassica rapa*, Cadmium, Organic acids, Oxidative stress, Phytohormones, Phytoremediation

1. Introduction

Heavy metals are important environmental pollutants that reduce crop productivity and are a threat to all the food chain including human health. One of the most toxic heavy metals is cadmium (Cd) being also dangerous due to its high mobility and availability for all living organisms. In plants, Cd produces severe toxicity symptoms including chlorosis, reduced growth, and impairment of physiological processes such as photosynthesis, respiration, transpiration and nutrient uptake (Clemens and Ma, 2016). Thus, Cd triggers reactive oxygen species (ROS) accumulation producing oxidative stress (Sandalio *et al*., 2009). ROS cause membrane destabilization due to lipid peroxidation (Sharma *et al*., 2012). In addition, ROS impair chlorophylls biosynthesis and photosystems

decreasing photosynthetic performance (D'Alessandro *et al*., 2013). To counteract ROS effects, plants induce antioxidant systems that maintain cellular redox homeostasis (Sharma *et al*., 2012). On the other hand, another effect of Cd stress is the changes in the concentration and distribution of phytohormones (Hu *et al*., 2013; Han *et al*., 2013). Likewise, the application of phytohormones such as indole-3-acetic acid (IAA), gibberellins (GAs) or cytokinins (CKs) could enhance Cd tolerance through a better antioxidant system and reducing Cd accumulation (Bashri and Prasad, 2016; Ahmad *et al*., 2015). Thus, phytohormones and the interaction among them could be crucial in the defense of plants against Cd stress (Han *et al*., 2013; Hu *et al*., 2013).

In order to avoid the toxic effect of Cd, plants increase the production of GSH and phytochelatins (PCs) and organic acids (OAs) such as citrate, malate, and oxalate. Due to their complexing properties, they form metal-organic complexes that mitigate the toxic effect of heavy metals. GSH plays a role in the elimination of H_2O_2 and lipid peroxides and in the expression of stress-responsive genes (Gill and Tujeta, 2010). Likewise, PCs and OAs act in the detoxification of heavy metal through direct chelation, facilitating its transport and storage in vacuoles and therefore preventing its toxic effect (Ryan *et al*., 2001; Grill *et al*., 2007). Thus, PCs and OAs could be useful in phytoremediation programs either because they increase their absorption and transport towards the shoot or because they increase their tolerance by sequestering the metal in vacuoles (Hawrylak-Nowak *et al*., 2015).

Phytoremediation is an environmentally friendly strategy to clean up soils contaminated with heavy metals (Azevedo *et al*., 2012). Species such as *Brassica rapa* are suitable for Cd phytoremediation as they tolerate relatively high levels of Cd and accumulate it in a concentration up to 100 ppm (Baker *et al*., 2000; D'Alessandro *et al.*, 2013). Researchers are generating and selecting new varieties with enhanced phytoremediation capacity through the modification of certain genes and protein expression (Ali-Zade *et al*., 2010). Potential targets are CAXs transporters (Baliardini *et al*., 2015). CAXs are a family of Ca^{2+}/H^{+} antiporters located on plasma and organelle membranes that play a key role in Ca homeostasis and in signalling processes driven by Ca (Pittman and Hirschi, 2016). CAXs modification could have a great impact on Cd tolerance due to Cd and Ca are antagonistic elements, both elements have similar physical features and they compete with each other for their entry into the plants (Tran and Popova, 2014). In addition, Ca can indirectly alleviate the toxic effects of Cd due to its relation to ROS production (Rodríguez-Serrano *et al*., 2009). Finally, the most important reason is that CAX1 is able to transport Cd inside the vacuole. Thus, in two independent experiments, Shigaki *et al*. (2005) and Wu *et al*. (2011) obtained a CAX1 variant named CAXcd with enhanced Cd transport capacity.

TILLING (Targeting Induced Local Lesions in Genomes) technique is a potential tool to obtain new variants of CAX1 transporter. This technique allows the screening of new variations in target genes that may be useful for the development of plants with improved characteristics (Till, 2003). One of these improvements could be an enhanced phytoremediation capacity. Three mutants of *B. rapa* ssp. trilocularis 'R-o-18' CAX1a transporter were generated using the

TILLING technique: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12* (Lochlainn *et al*., 2011). These mutations change amino acids (AAs) upstream of the N-terminal autoinhibitory domain, but they could change protein conformation and affect CAX1 function (Graham *et al*., 2014). In addition, it was observed recently that these mutations produced changes in some primary metabolic processes and in Ca accumulation (Navarro-León *et al*., 2019). These changes could provide tolerance against some stress such as heavy metal toxicity. Therefore, the objective of this work is to evaluate the tolerance of these mutants to Cd toxicity and to evaluate its potential for phytoremediation purposes.

2. Material and methods

2.1. Plant material, growth conditions, and treatments

Three mutants of *Brassica rapa* L. subsp. *trilocularis* (Roxb.) genotype R-o-18 (*BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56)) and the parent line R-o-18 were employed as plant material for the experiment. Mutant plants were obtained and identified as described by Lochlainn *et al*. (2011) and Graham *et al*. (2014). Seeds were sown on filter paper moistened with milli-Q water (18.2 MV cm) in 9 cm Petri dishes. The dishes were sealed with plastic film and incubated in the dark for 1 d at 4ºC before transferring to pots filled with vermiculite. These pots where placed in a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 22/18ºC (day/night) and 14/10-h photoperiod at a photosynthetic photon flux

density of 350 µmol m^{-2} s^{-1} (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Throughout the experiment the plants received a modified Hoagland's solution composed of 4 mM KNO_3, 4 mM $Ca(NO_3)_2 \cdot 4\ H_2O$, 2 mM $MgSO_4 \cdot 7\ H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4 \cdot 2\ H_2O$, 2 µM $MnCl_2 \cdot 4\ H_2O$, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4 \cdot 5\ H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2\ H_2O$, 5 µM Fe-chelate (Sequestrene; 138FeG100), and 10 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was renewed every three days.

2.2. Experimental design and treatments

Treatments were started 30 days after germination and were kept for 21 days. Plants received two different treatments: Control (without $CdCl_2$) and Cd toxicity (100 µM $CdCl_2$). The applied $CdCl_2$ dose was selected based on the results of a previous experiment with the same plants grown with doses ranging from 0 µM to 250 µM (data not shown). The two factors involved in the experiment were the Cd treatment (C) and the mutant employed (M). The experimental design consisted of a randomized complete block with 12 treatments, eight plants per treatment and three replications each.

2.3. Plant sampling and determination of the relative growth rate (RGR)

Plant leaves were washed with distilled water, dried on filter paper, and weighed for the fresh weight (FW). Half of the leaves from each treatment were frozen at −30ºC for later biochemical assays and the other half of the plant material was lyophilized to measure the dry weight (DW) and the nutrient concentrations. To determine the RGR, leaves from each genotype were

sampled immediately before starting the $CdCl_2$ treatment (Ti). The leaves were dried in a forced-air oven at 70 °C for 24 h, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled after 21 days of treatment (Tf). The RGR values were calculated using the equation RGR = (ln DWf − ln DWi)/(Tf − Ti) where T is the time and the subscripts denote the final and initial sampling (i.e. days 0 and 21, respectively, after the beginning of the $CdCl_2$ treatment).

2.4. Analysis of cadmium, calcium and magnesium concentrations and cadmium distribution coefficient (DC)

Cd, calcium (Ca), and magnesium (Mg) were determined after a sample of 150 mg dry material was subjected to a process of mineralization by wet digestion (Wolf, 1982). Dry leaves were ground and mineralized with a mixture of nitric acid (HNO_3)/perchloric acid ($HClO_4$) (v/v) and H_2O_2 at 30%. From the resulting mineralization, and after the addition of 20 ml of mili-Q H_2O, elements concentrations were determined by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA).

DC was calculated as the quotient between Cd concentration in leaves and Cd concentration in roots (Zhu *et al*., 2003). Cd content was determined as the product of leaf DW and Cd concentration.

2.5. Pigment concentrations, SPAD chlorophyll value, and PI_{ABS}

Total chlorophyll and carotenoid were extracted in methanol and centrifuged at 5000 × *g* for 5 min. Thereafter, the absorbance of the supernatant was measured at 664, 648, and 470 nm. The chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) were estimated using the Lichtenthaler equation (Hartmut and Lichtenthaler, 1987). Total Chl was calculated as the sum of Chl *a* and Chl *b*.

The relative chlorophyll content of leaves was measured by using SPAD chlorophyll meter SPAD-502 (Konica Minolta Sensing Inc., Japan). Three measurements were made in each leaf and the average was calculated.

For PI_{ABS} measure, plants were adapted to dark for 30 min before measurements using special leaf clip holders that were allocated in each leaf. Chl *a* fluorescence kinetics was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK); the fluorescence transients were induced by red light (650 nm) with 3000 µmol photons $m^{-2}s^{-1}$ light intensity and recorded by the instrument. Measurements were conducted with six plants of fully expanded leaves at midstem position. Handy PEA software provides the values of performance index for energy conservation from photons absorbed by PSII antenna to the reduction of Q_B (PI_{ABS}) (Strasser *et al*., 2000).

2.6. ROS detoxification enzyme activities, $O_2^{\bullet-}$ and H_2O_2 concentrations and lipid peroxidation

Superoxide dismutase (SOD) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Yu *et al*. (1998). Catalase (CAT) activity was determined following the consumption of H_2O_2 at 240 nm (Nakano and Asada, 1981). The $O_2^{\cdot-}$ determination was based on the ability to reduce NBT. Optical density was measured at 580 nm (Kubiś, 2008). H_2O_2 concentration was colorimetrically measured as described by Junglee *et al*. (2014). Malondialdehyde (MDA) concentration was determined according to Fu and Huang (2001) measuring absorbance at 532 nm. The non-specific absorbance value at 600 nm was obtained to correct the turbidity. MDA concentration was calculated using 155 mM^{-1} cm^{-1} as the extinction coefficient.

2.7. Lipoxygenase (LOX), peroxidases and glutathione reductase (GR) activities

LOX activity was measured according to Minguez-Mosquera *et al*. (1993). Ascorbate peroxidase (APX) and glutathione reductase (GR) activities were assayed following Rao et al. (1996). APX activity was determined registering the absorbance change at 290 nm. GR activity was measured after monitoring the oxidation of NADPH at 340 nm for 3 min. Glutathione peroxidase (GPX) activity was measured as described by Elia *et al*. (2003) with slight modifications using H_2O_2 as a substrate and the oxidation of NADPH was recorded at 340 nm. The

protein concentration of extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

2.8. Glutathione (GSH) and ascorbate (AsA) assay

Oxidized GSH (GSSG), and total GSH (reduced GSH + GSSG) were analyzed according to Noctor and Foyer (1998). Reduced GSH levels were estimated as the difference between total GSH and GSSG. The extraction and quantification of total AsA, reduced AsA, followed the method of Law *et al*. (1983). The dehydroascorbate (DHA) concentration was deduced from the difference between total AsA and reduced AsA. Redox states of AsA and GSH were calculated using the formula: [(Reduced form) X 100]/ [Reduced + Oxidized forms].

2.9. Extraction and analysis of organic acids

Malic, citric and oxalic acids were analyzed according to Gómez-Romero *et al*. (2010) with some modifications. Briefly, 75 mg of freeze-dried and ground leaves were dropped in 1 ml of cold (-20°C) extraction mixture of methanol/water/acetic acid (80/19.5/0.5, v/v/v). The blend was centrifuged (20,000 *g*, 15 min) and re-extracted for 30 min at 4ºC in an additional 1 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C_{18} cartridges (SepPak Plus, Waters, USA) and evaporated at 40ºC under vacuum to near dryness. The residue was dissolved in 1 ml water/methanol/acetic acid (94.5/5/0.5, v/v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with

0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA). 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The analytes were separated using a Zorbax SB-C18 HPLC column (5 µm, 150 x 0.5 mm, Agilent Technologies, Santa Clara, CA, USA), maintained at 30 ºC. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For OAs quantification, calibration curves were constructed for each analyzed component (1, 2.5, 5, and 10 mg l^{-1}).

2.10. Tricarboxylic acids (TCA) enzyme extractions and assays

Extracts for measuring enzyme activities were made following the method of Li (2000). Citrate synthase (CS) activity was assayed spectrophotometrically by monitoring the reduction of acetyl coenzyme A (CoA) to Co A with 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm (Srere, 1969). Phosphoenolpyruvate carboxykinase (PEPC) activity was measured according to López-Millán *et al*. (2001). Finally, MDH activity was determined with oxalate as substrate by measuring the decrease in absorbance at 340 nm due to the enzymatic oxidation of NADH (Dannel *et al*., 1995).

2.11. Hormone extraction and analysis

IAA, GAs (GA1, GA3, and GA4), CKs (trans-Zeatin (tZ) and isopentenyl-adenine (iP)), ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and ABA (abscisic acid) were analyzed as in Ghanem *et al*. (2008) with some

modifications. Briefly, 30 mg of homogenized dry material were dropped in 0.5 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20,000 *g*, 15 min) and re-extracted for 30 min at 4ºC in additional 0.5 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, USA) and evaporated at 40ºC under vacuum. The residue was dissolved in 1 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through Millex nylon membrane filters 13 mm diameter of 0.22 µm pore size (Millipore, Bedford, MA, USA). Next, 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The mass spectra were determined using the Xcalibur software version 2.2. For quantification of plant hormones, calibration curves were constructed for each component analyzed (1, 10, 50, and 100 µg l^{-1}) and corrected for 10 µg l^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%.

2.12. Statistical analysis

Data were subjected to a simple ANOVA at 95% confidence. A two-tailed ANOVA was applied to ascertain whether the Cd treatment (C), the mutations (M), or the interaction (C * M) significantly affected the results. Means were compared by Fisher's least significant differences (LSD). Linear correlation coefficients between leaf Cd and leaf Ca or Mg concentration were obtained. The

significance levels for both analyses were expressed as * P<0.05, ** P<0.01, *** P<0.001, or NS (not significant). All statistical analyses were carried out using the Statgraphics Centurion XVI software.

3. Results

3.1. Biomass and relative growth rate

The application of 100 µM $CdCl_2$ to *B. rapa* plants caused a substantial decrease in leaf biomass in comparison to control conditions. However, *BraA.cax1a-12* plants presented higher leaf DW than the other lines. With respect roots DW, Cd affects it in a lesser than leaves. Comparing lines, only *BraA.cax1a-7* presented a lower root DW value in comparison to R-o-18 plants. Finally, under Cd toxicity conditions, *BraA.cax1a-12* registered the highest foliar RGR values, followed by *BraA.cax1a-7* and, lastly, *BraA.cax1a-4* presenting similar value than R-o-18 plants (Table 1).

Table 1. Leaf and root growth and leaf Ca concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to Cd toxicity.

		Leaf biomass (g DW plant^{-1})	Root biomass (g DW plant^{-1})	Leaf RGR (g g^{-1} day^{-1})
Control	R-o-18	0.71±0.04a	0.19±0.02a	0.13±0.01c
	BraA.cax1a-4	0.62±0.04a	0.18±0.01a	0.14±0.01b
	BraA.cax1a-7	0.65±0.34a	0.09±0.01b	0.16±0.01a
	BraA.cax1a-12	0.65±0.03a	0.13±0.02b	0.16±0.01a
	p-value	NS	***	***
	$LSD_{0.05}$	0.09	0.04	0.01
100 µM $CdCl_2$	R-o-18	0.30±0.02b	0.14±0.01ab	0.09±0.01c
	BraA.cax1a-4	0.25±0.03b	0.10±0.01bc	0.10±0.01c
	BraA.cax1a-7	0.28±0.03b	0.08±0.01c	0.12±0.01b
	BraA.cax1a-12	0.46±0.03a	0.15±0.02a	0.13±0.00a
	p-value	***	**	***
	$LSD_{0.05}$	0.07	0.04	0.01
Analysis of variance				
Cd (C)		***	**	***
Mutation (M)		**	***	***
C x M		**	**	**
$LSD_{0.05}$		0.06	0.03	0.01

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.2. Cd concentration, content and distribution coefficient

Both *BraA.cax1a-7* and *BraA.cax1a-12* mutations increased Cd concentration and content in leaves, although this increase was greater in *BraA.cax1a-12* plants presenting 104% higher Cd concentration and 212% higher Cd content than R-o-18 plants (Fig. 1A y 1D). On the other hand, root Cd concentration was greater in all mutant lines, being again *BraA.cax1a-12* the mutant that registered the higher levels (Fig. 1B). On the other hand, *BraA.cax1a-*

12 and *BraA.cax1a-4* plants showed lower DC values in comparison to R-o-18 plants (Fig. 1C).

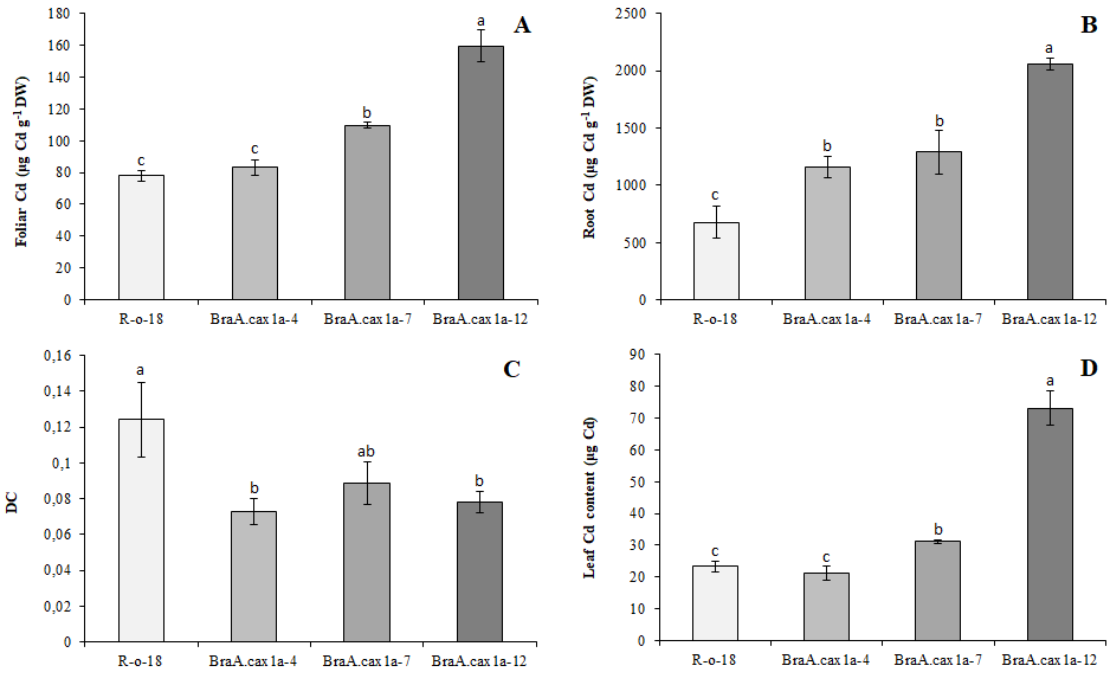

Figure 1. Foliar (A) and root (B) Cd concentration, Cd distribution coefficient (C) and leaf Cd content (D) in *BraA.cax1a* mutants and R-o-18 plants subjected to Cd toxicity. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

3.3. Ca and Mg concentrations

Cd toxicity did not decrease leaf Ca and Mg concentrations with respect control conditions. However, *BraA.cax1a* plants showed higher Ca and Mg concentrations in comparison to R-o-18 plants under Cd toxicity conditions. Thus, the highest Ca and Mg levels were reached in *BraA.cax1a-12* plants (Fig. 2). In addition, correlation analysis between leaf Cd and Ca concentration showed a positive correlation factor (0.85; $P < 0.001$) and a similar correlation factor was observed between leaf Cd and Mg concentration (0.83; $P < 0.001$).

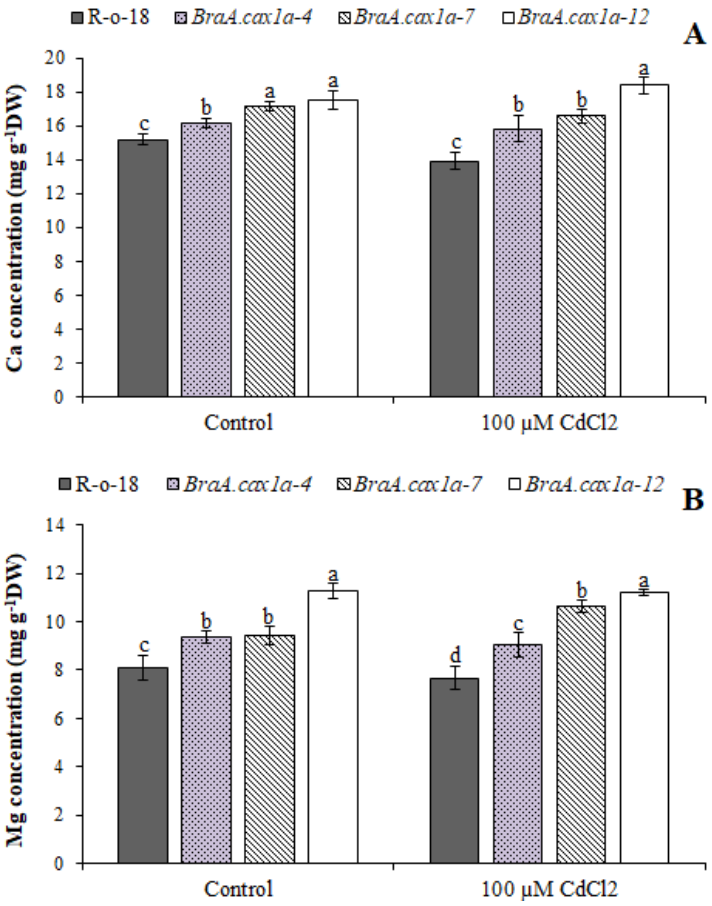

Figure 2. Ca (A) and Mg (B) concentrations in *BraA.cax1a* mutants and R-o-18 leaves of plants grown under Cd toxicity. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

3.4. Pigments concentration and photosynthetic performance

Under Cd toxicity conditions, *BraA.cax1a-4* and especially *BraA.cax1a-7* plants presented lower SPAD and PI_{ABS} values, whereas *BraA.cax1a-12* plants presented no significant differences in comparison to R-o-18 plants. On the other hand, *BraA.cax1a* mutations affected negatively to total Chl concentration. However, *BraA.cax1a* mutants presented higher Chl *a*/*b* ratio values in comparison to R-o-18 plants (Table 2).

Table 2. SPAD chlorophyll, total Chl concentration, Chl *a*/*b* ratio, and carotenoids concentration in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity

		SPAD chlorophyll	Total Chl mg g^{-1} FW	Chl *a*/*b*	PI_{ABS}
Control	R-o-18	42.44±2.38a	0.35±0.01a	2.34±0.02b	7.74±2.21a
	BraA.cax1a-4	30.08±2.81b	0.29±0.00b	2.63±0.10a	1.91±0.38c
	BraA.cax1a-7	29.52±2.90b	0.26±0.01c	2.63±0.07a	2.43±1.40c
	BraA.cax1a-12	40.06±1.63a	0.30±0.01b	2.71±0.09a	4.95±2.17b
	p-value	***	***	*	***
	$LSD_{0.05}$	2.61	0.01	0.22	2.15
100 µM $CdCl_2$	R-o-18	37.09±2.21a	0.43±0.01a	1.87±0.02b	5.70±1.98a
	BraA.cax1a-4	27.54±3.38c	0.37±0.01b	2.72±0.16a	1.07±0.29b
	BraA.cax1a-7	32.32±2.55b	0.24±0.01d	2.57±0.06a	1.51±0.85b
	BraA.cax1a-12	34.94±2.19a	0.29±0.01c	2.75±0.14a	5.43±1.25a
	p-value	***	***	***	***
	$LSD_{0.05}$	2.62	0.02	0.37	1.49
Analysis of variance					
Cd (C)		***	***	*	*
Mutation (M)		***	***	***	***
C x M		***	***	*	NS
$LSD_{0.05}$		1.81	0.01	0.21	1.26

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.5. Oxidative stress parameters and AsA-GSH cycle

Cd application caused an increase in $O_2^{\cdot-}$ levels and in LOX activity in comparison to control conditions. However, LOX increase was not significant in *BraA.cax1a-12*. With respect to MDA and H_2O_2 concentrations, when Cd was applied, we only observed an increase in R-o-18 and *BraA.cax1a-7* compared with control plants (Table S1). Comparing between lines, *BraA.cax1a-12* plants presented the lowest $O_2^{\cdot-}$, MDA, H_2O_2 concentrations and the highest LOX, SOD and CAT activities. Likewise, *BraA.cax1a-4* plants also presented lower H_2O_2 concentration and higher CAT activity in comparison to R-o-18 plants when they were grown with Cd (Fig. 3; Table S1).

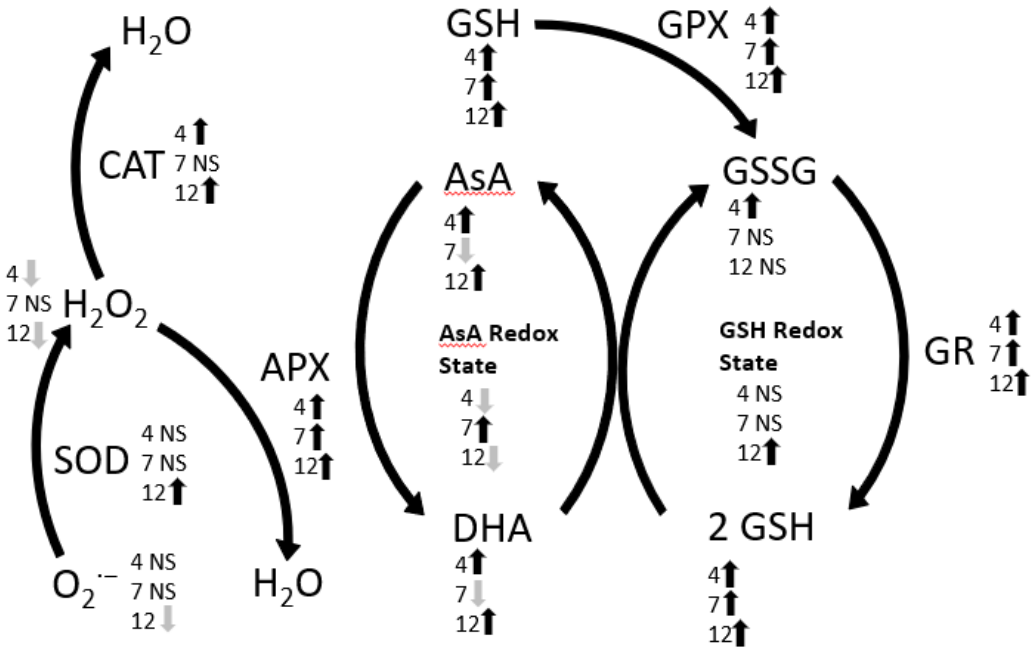

Figure 3. Scheme showing the differences between lines grown under Cd toxicity regarding the analyzed elements of the AsA/GSH cycle. Numbers indicate each *BraA.cax1a* mutant: *BraA.cax1a-4* (4), *BraA.cax1a-7* (7), and *BraA.cax1a-12* (12). Upward-pointing black arrows indicate an increment, downward-pointing grey arrows indicate a decrease, and NS indicate non-significant differences with respect R-o-18 plants.

3.6. Ascorbate and glutathione metabolism

In general, Cd stress reduced the levels of most of the AsA/GSH components in R-o-18. These plants presented lower GR, GPX activities and lower total GSH, GSH, total AsA, AsA and DHA concentrations in comparison to control conditions. In the case of *BraA.cax1a* mutants the reduction in GR activity was lower and presented no significant differences or increments in AsA/GSH cycle components (Table S2; S3; Fig. S1). Comparing between lines, all *BraA.cax1a* presented higher APX, GR and GPX activities, and higher total GSH, GSH concentrations, although *BraA.cax1a-4* reached the highest APX activity and *BraA.cax1a-12* the highest GPX activity. *BraA.cax1a-4* and *BraA.cax1a-12* showed higher total AsA, AsA and DHA concentrations in comparison to R-o-18, although *BraA.cax1a-12* reached the highest concentrations of these parameters (Fig. 3 and S1). Finally, *BraA.cax1a-7* showed lower AsA, AsA and DHA concentrations than R-o-18 but had a higher AsA redox state value (Fig. 3).

3.7. OAs and TCA cycle

Regarding malate concentration, the Cd application reduced its concentration in all lines except in *BraA.cax1a-12* that accumulated more of this OA in comparison to the other lines. With respect to citrate, Cd increased its concentration only in *BraA.cax1a-4* and *BraA.cax1a-7* plants and reduced it in *BraA.cax1a-12* plants in comparison to control conditions (Table 3). Comparing between lines, *BraA.cax1a-7* and *BraA.cax1a-12* plants presented lower citrate levels than R-o-18 plants. Finally, oxalate increased in *BraA.cax1a* mutants due to Cd application reaching higher levels than in R-o-18 plants (Table 3).

Cd toxicity caused inhibition of CS activity in comparison to control conditions. However, this inhibition was higher in *BraA.cax1a-7* plants and lower in *BraA.cax1a-12* plants that reached the highest CS activity. No differences between lines were observed regarding MDH activity. In contrast, PEPC activity was lower in *BraA.cax1a-7* plants and higher in *BraA.cax1a-4* and *BraA.cax1a-12* plants, although this last mutant reached the highest PEPC activity (Table 3).

Table 3. Organic acids concentration and activities of some TCA cycle enzymes in *BraA.cax1a* mutants
toxicity

		Malic acid (mg g^{-1}DW)	Citric acid (mg g^{-1}DW)	Oxalic acid (mg g^{-1}DW)	CS (ΔAbs mg protein^{-1} min^{-1})	MDH (ΔAbs mg $^{1}min^{-1}$)
Control	R-o-18	12.89±0.70b	9.09±0.83a	0.09±0.01a	0.21±0.02a	1.13±0.01
	BraA.cax1a-4	16.56±1.06a	5.08±0.55b	0.06±0.01b	0.22±0.02a	0.94±0.02
	BraA.cax1a-7	6.52±0.13c	3.92±0.05b	0.04±0.00c	0.21±0.05a	1.22±0.02
	BraA.cax1a-12	12.74±1.74b	9.15±0.55a	0.05±0.01b	0.19±0.03a	1.23±0.06
	p-value	**	***	***	NS	***
	$LSD_{0.05}$	3.52	1.64	0.01	0.09	0.10
100 µM $CdCl_2$	R-o-18	9.49±0.88b	8.36±0.66a	0.05±0.00c	0.10±0.01b	0.96±0.02
	BraA.cax1a-4	8.95±0.22b	9.34±0.35a	0.07±0.01b	0.10±0.01b	0.91±0.09
	BraA.cax1a-7	5.69±0.09c	5.59±0.19b	0.10±0.01a	0.07±0.01c	1.00±0.04
	BraA.cax1a-12	16.37±0.24a	6.25±0.01b	0.08±0.01b	0.15±0.01a	0.88±0.12
	p-value	***	***	***	***	NS
	$LSD_{0.05}$	1.54	1.26	0.01	0.02	0.12
Analysis of variance						
Cd (C)		**	NS	***	***	***
Mutation (M)		***	***	NS	NS	***
D x M		***	***	***	NS	***
$LSD_{0.05}$		1.77	0.95	0.01	0.04	0.07

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance
letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.

3.8. Phytohormone profile

Cd affected differently to IAA depending on the line. IAA levels increased in R-o-18 plants, did not show significant changes in *BraA.cax1a-4* plants and decreased in *BraA.cax1a-7* and *BraA.cax1a-12* in comparison to control conditions. However, comparing between lines, IAA concentration only decreased in *BraA.cax1a-7* plants whereas the rest of the lines presented similar IAA values. *BraA.cax1a-12* plants presented the highest GA4 levels, while the rest of the mutants showed similar levels than R-o-18 plants. With respect to total GAs, they were lower in *BraA.cax1a-4* and *BraA.cax1a-7* plants and showed no differences in *BraA.cax1a-12* in comparison to R-o-18 plants. Cd application decreased Ip concentration compared to control plants in all lines. Comparing between lines, iP was lower in *BraA.cax1a-4* and *BraA.cax1a-7* plants whereas tZ concentration was lower in *BraA.cax1a-7* and *BraA.cax1a-12* plants. Thus, total CKs decreased only in *BraA.cax1a-12* plants with respect to R-o-18 plants. Regarding stress-related hormones such as ABA and ACC, Cd application increased greatly ABA concentration in R-o-18 plants and ACC in all lines. Comparing between lines, *BraA.cax1a* present lower levels of these two hormones, presenting *BraA.cax1a-12* the lowest ACC levels (Table 4).

Table 4 Phytohormones concentration in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity.

		IAA	GA1	GA3	GA4	GAs	iP	tZ	
Control	R-o-18	7.03±0.36c	0.08±0.02a	Nd	0.02±0.01a	0.10±0.01ab	2.88±0.25a	203±15ab	2a
	BraA.cax1a-4	12.61±1.34b	0.04±0.01ab	Nd	0.01±0.01a	0.05±0.01b	1.45±0.21b	167±8b	1
	BraA.cax1a-7	10.52±1.74bc	0.08±0.02a	0.04±0.01	0.01±0.01a	0.14±0.02a	1.26±0.27b	115±1c	1
	BraA.cax1a-12	18.58±1.11a	0.01±0.00b	0.07±0.02	0.02±0.01a	0.10±0.02ab	2.55±0.17a	231±16a	2a
	p-value	**	*		NS	*	**	***	*
	$LSD_{0.05}$	4.06	0.05		0.01	0.06	0.75	38	3
100 µM $CdCl_2$	R-o-18	12.94±1.67a	0.08±0.02	0.02±0.01	0.01±0.01b	0.09±0.01a	3.57±0.44a	215±15a	2a
	BraA.cax1a-4	10.94±1.56ab	Nd	Nd	0.02±0.01b	0.02±0.01b	1.72±0.06b	191±6ab	1
	BraA.cax1a-7	7.54±0.33b	0.03±0.01	Nd	0.02±0.01b	0.03±0.01a	1.96±0.17b	178±9b	2a
	BraA.cax1a-12	13.17±0.31a	Nd	0.02±0.01	0.05±0.01a	0.08±0.02a	2.96±0.06a	110±5c	1
	p-value	*			**	*	**	***	*
	$LSD_{0.05}$	3.79			0.02	0.04	0.87	31	5
Analysis of variance									
Cadmium (C)		*			**	**	**	*	*
Mutation (M)		***			***	**	***	***	*
C x M		**			*	*	NS	***	*
$LSD_{0.05}$		2.55			0.01	0.03	0.50	22.50	2

Values are means ± standard error (n=9) expressed in ng g^{-1} DW and differences between means were compared b
P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p
p<0.01 (**) and p<0.001 (***).

4. Discussion

4.1. Plant biomass and RGR

The negative effect of Cd on plant biomass and growth has been thoroughly studied (D'Alessandro *et al*., 2013; Srivastava *et al*., 2015). Thus, in *Brassica juncea* plants the application of 100 μM $CdCl_2$ caused a 30% biomass reduction (D'Alessandro *et al*., 2013). In the present experiment, we measured roughly a 60% decrease of leaf biomass in all the genotypes except in *BraA.cax1a-12* were the decrease was only 30%. This mutant also presented the highest RGR values (Table 1). Therefore, *BraA.cax1a-12* mutation provides a higher tolerance to Cd toxicity. This higher Cd tolerance could be due to a better Ca homeostasis under Cd stress conditions. Indeed, Ca has been proved as a Cd antagonism mitigating Cd toxic effects (Srivastava *et al*., 2015).

4.2. Cd accumulation

In the present experiment, a**s** it was observed in other similar experiments (Cho *et al*., 2012 and references therein), the major proportion of applied Cd remained in the root. Therefore, which determines a better phytoremediation efficiency is the capacity to transport the heavy metal to the aerial tissues (Ali-Zade *et al*., 2010). According to our results, *BraA.cax1a* mutations provide a higher Cd uptake capacity because all mutants accumulated more Cd in roots than parental line R-o-18 (Fig.

1B). Likewise, in the case of *BraA.cax1a-7* and *BraA.cax1a-12,* it provided higher Cd accumulation capacity in the shoot (Fig. 1A). However, *BraA.cax1a* mutation did not provide higher translocation capacity to the shoot as they presented lower DC values than R-o-18 plants (Fig. 1C). Despite this, *BraA.cax1a-12* could be highlighted as the mutant with better phytoremediation efficiency as it presented more than three-fold more Cd content than R-o-18 plants (Fig. 1D). The different Cd accumulation capacity in mutants could be due to the well-known antagonistic relationship between Ca, Mg and Cd (Srivastava *et al.*, 2015) and the effect of *BraA.cax1a* on the accumulation of these elements (Navarro-León *et al.*, 2018). Surprisingly, we did not observe the antagonism between Ca, Mg and Cd. Indeed, we observed positive correlations between Cd and the other cations and *BraA.cax1a-12* was the mutant with higher Ca and Mg concentrations under Cd toxicity (Fig. 2). This could serve as protection against Cd toxicity and could explain in part the higher Cd tolerance in this mutant. Other studies showed that Ca and Mg accumulation could be effective to avoid Cd toxicity (Zorrig *et al*., 2010; Tran and Popova, 2014). In addition, *BraA.cax1a-7* and *BraA.cax1a-12* through modification in the Ca compartmentalization could induce Cd uptake, probably by reducing Ca cytosol levels which would allow a higher Cd uptake.

4.4. Pigment and photosynthesis

Cd causes a negative effect on Chl concentration and in photosynthesis performance (D'Alessandro *et al.*, 2013). However, in the present work, Cd only reduced total Chl concentration in *BraA.cax1a-4* and *BraA.cax1a-7* lines with respect to control conditions (Table 2). Although this reduction was not enough to affect negatively to plant biomass (Table 1). In spite of Chls reduction, *BraA.cax1a* mutations presented a greater Chl *a*/ Chl *b* (Table 2). Chl *a* is principally associated with reaction centers and core antenna proteins of PSII while Chl *b* is principally localized in light-harvesting complexes (LHCII). Therefore Chl *a*/*b* reflects RC/LHCII proportion, when this ratio declines, usually is linked to a reduction of photosynthetic capacity (Nyongesah *et al*., 2015). Therefore, *BraA.cax1a* under Cd toxicity could favor the conversion of Chl *b* to Chl *a*, in order to maintain Chl *a* levels, and thereby active RCs. In addition, Cd compromise photosynthesis performance inducing conformational changes in LHCII and OEC and interfere with electron transport (D'Alessandro *et al*., 2013). In our experiment, this negative effect of Cd is clearly observed in R-o-18 plants that presented a reduction in the PI_{ABS} parameter in comparison to control conditions. However, this parameter was not affected by Cd in *BraA.cax1a-12* (Table 2). D'Alessandro *et al*. (2013) proved the protective role of Ca against Cd toxicity. Thus, a greater Ca concentration and homeostasis could protect photosynthetic systems against Cd toxicity in *BraA.cax1a-12* mutant.

4.5. Oxidative metabolism and AsA/GSH cycle

One of the most known effects of Cd toxicity is oxidative stress and the subsequent production of ROS and MDA (Sandalio et al., 2009). Likewise, Cd usually inhibits the activity of some antioxidant enzymes such as SOD, APX, and CAT contributing, in turn, to a higher ROS accumulation (Srivastava *et al*., 2015). As in the majority of studies, we found higher $O_2^{\cdot -}$ levels in all plants subjected to Cd stress. However, MDA and H_2O_2 levels did not increase in *BraA.cax1a-4* and *BraA.cax1a-12* plants and LOX activity did not increase in *BraA.cax1a-12* plants (Fig. 3; Table S1). These two mutants probably possess an improved H_2O_2 detoxification machinery through higher CAT and APX activities (Fig. 3). On the other hand, *BraA.cax1a-12* presented lower $O_2^{\cdot -}$ levels than the other lines which is probably by a higher SOD activity (Fig. 3). High cytosolic Ca levels promote ROS production and decrease of SOD activity under Cd toxicity (Srivastava *et al*., 2015). Therefore, in *BraA.cax1a-12* plants an enhanced CAX1 activity could reduce ROS levels and allow a greater Cd tolerance.

AsA/GSH cycle is one of the main ways to recycle antioxidants in plants and to eliminate ROS maintaining the reduced state in cells that usually is loss under metal stress (Gill and Tujeta, 2010; Sharma et al., 2012). The effect of Cd application on the AsA/GSH cycle can be either positive or negative depending on the species (Gill and Tujeta, 2010; D'Alessandro *et al*., 2013; Han *et al.*, 2013). In the present

experiment, compared with control conditions, Cd affected negatively to AsA/GSH cycle components in R-o-18 plants but not in *BraA.cax1a* plants (Table S2, S3). Comparing between lines, we observed that *BraA.cax1a-4* and *BraA.cax1a-12* presented an enhanced AsA/GSH cycle under Cd stress in comparison to R-o-18 (Fig. 3). However, this response was greater in *BraA.cax1a-12* plants as they showed higher AsA, GPX activity, and a greater GSH redox state. *BraA.cax1a-7* also appears to have a positive response on GSH part of the cycle although it presented a depletion in AsA levels (Fig. 3, S1; Table S2, S3). These effects of *BraA.cax1a* probably are produced by changes in Ca homeostasis. Thus, it was proved that GSH and the GSH/GSSG ratio is affected by Ca levels as it is reduced under Ca deficiency (Cho et al., 2012, and references therein). Ca also promotes GR and AsA-GSH cycle in Cd treated plants (Srivastava *et al*., 2015).

4.6. OAs and TCA cycle

Several studies proved that OAs such as malate, citrate, and oxalate can bind to heavy metals and increase the metal transport through the xylem and the vacuolar metal sequestration contributing to heavy metal tolerance (Dresler *et al*., 2014; Clemens and Ma, 2016). However, depending on the species analyzed, a different organic acid predominates (Sun et al., 2006; Dresler *et al.*, 2014). In the present experiment, we observed different responses of malate and citrate concentrations

depending on the line (Table 3). Thus, in *BraA.cax1a-12* Cd induces malate accumulation what could ease Cd accumulation and tolerance in this mutant. In the rest of the mutants, this malate response was not observed (Table 3) and they accumulated less Cd in shoots (Fig. 1A). Likewise, Han *et al*. (2006) observed that malate increased Cd uptake in maize plants and enhanced the accumulation in leaves. Regarding citrate, its effects on heavy metal uptake depend on the species (Evangelou *et al*., 2007). Citrate was not responsible for higher Cd accumulation and tolerance in *BraA.cax1a-12* plants. Finally, with respect to oxalate, there is a positive response of this OA to Cd in *BraA.cax1a* mutants (Table 3). Oxalate could contribute to higher Cd accumulation however it is less important than malate due to its lower concentration in plants (Wang *et al*., 1991).

In general, Cd toxicity increased TCA cycle enzyme activities (Willick, 2013). Nevertheless, in the present experiment, we observed a negative effect of Cd toxicity on CS activity in all plants in comparison to control conditions (Table 3). Although, *BraA.cax1a-12* plants maintained a higher CS activity which could contribute to higher Cd tolerance (Table 3). Considering the overall TCA activity, we observe three different responses depending on the *BraA.cax1a* mutant. The *BraA.cax1a-4* mutation did not produce changes in TCA enzyme activities except for a slight increase in PEPC activity. *BraA.cax1a-7* affected negatively to TCA cycle activity. In

contrast, *BraA.cax1a-12* mutation improved CS and PEPC activities (Table 3) which could provide a higher Cd tolerance. Because MDH activity did not increase in *BraA.cax1a-12* mutant (Table 3), this mutation could enhance the PEPC pathway as an alternative pathway to produce oxalate.

4.7. Phytohormone profile

Hu *et al*. (2013) and Lin et al. (2018) studies observed that Cd affects negatively to auxin biosynthesis and transport and induce oxidases that degrade auxin and GAs. However, the increase in growth-promoting hormones level appears to be positive in Cd tolerance *(*Ahmad *et al*., 2015). In the present study, no clear effect of Cd on IAA concentration is observed because Cd affected differently to each line and all lines presented similar IAA levels except for a decrease in *BraA.cax1a-7* plants (Table 4). Elobeid *et al*. (2012) showed that IAA affects Cd uptake in plant roots. However, in our study, we did not observe any relationship between Cd uptake and IAA concentration. Regarding GAs, it highlights an increase in GA4 in *BraA.cax1a-12* caused by the Cd application. This mutant presented the highest GA4 levels (Table 4), so GA4 could help to maintain growth in *BraA.cax1a-12* mutant. Likewise, the GA3 application promoted Cd accumulation in *Cannabis sativa* (Ahmad *et al*., 2015). Thus, GA4 may help to increase also Cd concentration in *BraA.cax1a-12* mutant. On the other hand, Ip concentration considerably increased

under Cd stress in comparison to control conditions (Table 4). The same result was observed by Cai *et al*. (2015) in rice plants. In contrast, Cd decreased CKs concentration only in *BraA.cax1a-12* (Table 4), which in theory could decrease the growth, although this is not produced.

Regarding stress-related phytohormones such as ABA, researchers measured increments in this hormone under Cd toxicity (Han *et al*., 2013). In contrast, the ABA application is able to reduce Cd accumulation and enhance Cd tolerance in rice seedling (Hsu and Kao, 2005). On the other hand, ethylene, a gaseous hormone synthesized from ACC, increases its synthesis when plants are under stress (Iqbal *et al*., 2013). Ethylene is able to trigger itself cell mortality induced by Cd as a consequence of a high ROS accumulation (Han *et al*., 2013). In the present experiment, it was observed a burst of ABA and ethylene levels in R-o-18 plants as a consequence of Cd toxicity (Table 4). However, *BraA.cax1a* plants somehow limit this phytohormone response and keep similar ABA values than in control conditions and a lesser increase of ethylene precursor ACC. With respect to ACC, *BraA.cax1a-12* plants presented lower levels than the other lines (Table 4) which could indicate less stress in this mutant.

5. Conclusions

According to the obtained results, all *BraA.cax1a* mutations provide greater Cd uptake capacity but do not increase Cd transport to the shoot. However, *BraA.cax1a-7* and especially *BraA.cax1a-12* mutations provide a greater Cd accumulation in leaves with respect R-o-18. This last mutant registered the greatest phytoextraction capacity with more than three-fold the Cd content of R-o-18. In spite of this, *BraA.cax1a-12* presents greater tolerance to Cd toxicity as it maintains a better growth than the other lines under Cd toxicity. Regarding the physiological response to Cd, it differs depending on the *BraA.cax1a* mutation. *BraA.cax1a-4* presents a similar response as R-o-18 plants although it has higher H_2O_2 detoxification capacity through AsA/GSH cycle. *BraA.cax1a-7* registered a more negative response because it shows lower AsA and OAs concentrations and lower TCA activity. On the other hand, in *BraA.cax1a-12* the greater Cd tolerance could be due to the higher Ca and Mg accumulations, the maintaining of photosynthesis performance, the enhanced ROS detoxification and AsA/GSH and TCA cycle, the higher malate, and GA4 concentrations and the lower ethylene levels. Briefly, this study identifies *BraA.cax1a-12* as a potential mutant for phytoremediation of Cd contaminated soils and identifies possible physiological elements that contribute to this capacity.

Acknowledgments

We thank Dr. Martin R. Broadley and Dr. Neil Graham from Nottingham University for providing us the seeds employed in this experiment

Funding

This work was supported by the PAI program (Plan Andaluz de Investigación, Grupo de Investigación AGR282) and by a Grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL [FPU14/01858].

References

Ahmad, A., Hadi, F., Ali, N., 2015. Effective phytoextraction of cadmium (Cd) with increasing concentration of total phenolics and free proline in *Cannabis sativa* (L) plant under various treatments of fertilizers, plant growth regulators and sodium salt. Int. J. Phytoremediation 17, 56–65. https://doi.org/10.1080/15226514.2013.828018

Ali-Zade, V., Alirzayeva, E., Shirvani, T., 2010. Plant Resistance to Anthropogenic Toxicants: Approaches to Phytoremediation, in: Plant Adaptation and Phytoremediation. Springer Netherlands, Dordrecht, pp. 173–192. https://doi.org/10.1007/978-90-481-9370-7_9

Ali, B., Gill, R.A., Yang, S., Gill, M.B., Ali, S., Rafiq, M.T., Zhou, W., 2014. Hydrogen sulfide alleviates cadmium-induced morpho-physiological and ultrastructural

changes in *Brassica napus*. Ecotoxicol. Environ. Saf. 110, 197–207. https://doi.org/10.1016/j.ecoenv.2014.08.027

Azevedo, R.A., Gratão, P.L., Monteiro, C.C., Carvalho, R.F., 2012. What is new in the research on cadmium-induced stress in plants? Food Energy Secur. 1, 133–140. https://doi.org/10.1002/fes3.10

Baker, A., McGrath, S., Reeves, R., Smith, J., 2000. Metal Hyperaccumulator Plants: A Review of the Ecology and Physiology of a Biological Resource for Phytoremediation of Metal-Polluted Soils, in: Terry, N., Bañuelos, G. (Eds.), Phytoremediation of Contaminated Soil and Water. Lewis Publisher, pp. 85–107

Baliardini, C., Meyer, C.-L., Salis, P., Saumitou-Laprade, P., Verbruggen, N., 2015. CATION EXCHANGER1 cosegregates with cadmium tolerance in the metal hyperaccumulator *Arabidopsis halleri* and plays a role in limiting oxidative stress in *Arabidopsis* Spp. Plant Physiol. 169, 549–59. https://doi.org/10.1104/pp.15.01037

Bashri, G., Prasad, S.M., 2016. Exogenous IAA differentially affects growth, oxidative stress and antioxidants system in Cd stressed *Trigonella foenum-graecum* L. seedlings: Toxicity alleviation by up-regulation of ascorbate-glutathione cycle. Ecotoxicol. Environ. Saf. 132, 329–338. https://doi.org/10.1016/j.ecoenv.2016.06.015

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Cai, B.-D., Yin, J., Hao, Y.-H., Li, Y.-N., Yuan, B.-F., Feng, Y.-Q., 2015. Profiling of phytohormones in rice under elevated cadmium concentration levels by magnetic solid-phase extraction coupled with liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1406, 78–86. https://doi.org/10.1016/j.chroma.2015.06.046

Cho, S.-C., Chao, Y.-Y., Kao, C.H., 2012. Calcium deficiency increases Cd toxicity and Ca is required for heat-shock induced Cd tolerance in rice seedlings. J. Plant Physiol. 169, 892–898. https://doi.org/10.1016/j.jplph.2012.02.005

Clemens, S., Ma, J.F., 2016. Toxic heavy metal and metalloid accumulation in crop plants and foods. Annu. Rev. Plant Biol. 67, 489–512. https://doi.org/10.1146/annurev-arplant-043015-112301

D'Alessandro, A., Taamalli, M., Gevi, F., Timperio, A.M., Zolla, L., Ghnaya, T., 2013. Cadmium stress responses in *Brassica juncea*: Hints from proteomics and metabolomics. J. Proteome Res. 12, 4979–4997. https://doi.org/10.1021/pr400793e

Dannel, F., Pfeffer, H., Marschner, H., 1995. Isolation of apoplasmic fluid from sunflower leaves and its use for studies on influence of nitrogen supply on apoplasmic pH. J. Plant Physiol. 146, 273–278. https://doi.org/10.1016/S0176-1617(11)82053-5

Dresler, S., Hanaka, A., Bednarek, W., Maksymiec, W., 2014. Accumulation of low-molecular-weight organic acids in roots and leaf segments of *Zea mays* plants treated with cadmium and copper. Acta Physiol. Plant. 36, 1565–1575. https://doi.org/10.1007/s11738-014-1532-x

Elia, A.C., Galarini, R., Taticchi, M.I., Dörr, A.J.M., Mantilacci, L., 2003. Antioxidant responses and bioaccumulation in *Ictalurus melas* under mercury exposure. Ecotoxicol. Environ. Saf. 55, 162–7. https://doi.org/https://doi.org/10.1016/S0147-6513(02)00123-9

Elobeid, M., Göbel, C., Feussner, I., Polle, A., 2012. Cadmium interferes with auxin physiology and lignification in poplar. J. Exp. Bot. 63, 1413–1421. https://doi.org/10.1093/jxb/err384

Evangelou, M.W.H., Ebel, M., Schaeffer, A., 2007. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. Chemosphere 68, 989–1003. https://doi.org/10.1016/j.chemosphere.2007.01.062

Fu, J., Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45, 105–114. https://doi.org/10.1016/S0098-8472(00)00084-8

Ghanem, M.E., Albacete, A., Martínez-Andújar, C., Acosta, M., Romero-Aranda, R., Dodd, I.C., Lutts, S., Pérez-Alfocea, F., 2008. Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.). J. Exp. Bot. 59, 3039–50. https://doi.org/10.1093/jxb/ern153

Gill, S.., Tujeta, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Gómez-Romero, M., Segura-Carretero, A., Fernández-Gutiérrez, A., 2010. Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. Phytochemistry 71, 1848–64. https://doi.org/10.1016/j.phytochem.2010.08.002

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of Brassica under altered Ca supply identifies *Arabidopsis* Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Grill, E., Mishra, S., Srivastava, S., Tripathi, R.D., 2007. Role of Phytochelatins in Phytoremediation of Heavy Metals, in: Environmental Bioremediation Technologies. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 101–146. https://doi.org/10.1007/978-3-540-34793-4_5

Han, F., Shan, X., Zhang, S., Wen, B., Owens, G., 2006. Enhanced cadmium accumulation in maize roots—the impact of organic acids. Plant Soil 289, 355–368. https://doi.org/10.1007/s11104-006-9145-9

Han, R.-M., Lefèvre, I., Albacete, A., Pérez-Alfocea, F., Barba-Espín, G., Díaz-Vivancos, P., Quinet, M., Ruan, C.-J., Hernández, J.A., Cantero-Navarro, E., Lutts, S., 2013. Antioxidant enzyme activities and hormonal status in response to Cd stress in the wetland halophyte *Kosteletzkya virginica* under saline conditions. Physiol. Plant. 147, 352–368. https://doi.org/10.1111/j.1399-3054.2012.01667.x

Hartmut, A., Lichtenthaler, K., 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382. https://doi.org/10.1016/0076-6879(87)48036-1

Hawrylak-Nowak, B., Dresler, S., Matraszek, R., 2015. Exogenous malic and acetic acids reduce cadmium phytotoxicity and enhance cadmium accumulation in roots of sunflower plants. Plant Physiol. Biochem. 94, 225–234. https://doi.org/10.1016/j.plaphy.2015.06.012

Hsu, Y.T., Kao, C.H., 2005. Abscisic acid accumulation and cadmium tolerance in rice seedlings. Physiol. Plant. 124, 71–80. https://doi.org/10.1111/j.1399-3054.2005.00490.x

Hu, Y.F., Zhou, G., Na, X.F., Yang, L., Nan, W. Bin, Liu, X., Zhang, Y.Q., Li, J.L., Bi, Y.R., 2013. Cadmium interferes with maintenance of auxin homeostasis in *Arabidopsis* seedlings. J. Plant Physiol. 170, 965–975. https://doi.org/10.1016/j.jplph.2013.02.008

Iqbal, N., Trivellini, A., Masood, A., Ferrante, A., Khan, N.A., 2013. Current understanding on ethylene signaling in plants: the influence of nutrient availability. Plant Physiol. Biochem. 73, 128–38. https://doi.org/10.1016/j.plaphy.2013.09.011

Kubiś, J., 2008. Exogenous spermidine differentially alters activities of some scavenging system enzymes, H_2O_2 and superoxide radical levels in water-stressed cucumber leaves. J. Plant Physiol. 165, 397–406. https://doi.org/10.1016/j.jplph.2007.02.005

Law, M.Y., Charles, S.A., Halliwell, B., 1983. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. The effect of hydrogen peroxide and of Paraquat. Biochem. J. 210, 899–903. https://doi.org/10.1042/BJ2100899

Li, X.F., 2000. Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. Plant Physiol. 123, 1537–1544. https://doi.org/10.1104/pp.123.4.1537

Lin, H., He, J., Lin, Weiwei, Li, Y., Fang, C., Lin, Wenxiong, 2018. Lsi1-regulated Cd uptake and phytohormones accumulation in rice seedlings in presence of Si. Plant Growth Regul. 86, 149–157. https://doi.org/10.1007/s10725-018-0417-1

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R., 2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

López-Millán, A.F., Morales, F., Abadía, A., Abadía, J., 2001. Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (*Beta vulgaris*) leaves. Physiol. Plant. 112, 31–38. https://doi.org/10.1034/j.1399-3054.2001.1120105.x

Minguez-Mosquera, M.I., Jaren-Galan, M., Garrido-Fernandez, J., 1993. Lipoxygenase activity during pepper ripening and processing of paprika. Phytochemistry 32, 1103–1108. https://doi.org/10.1016/s0031-9422(00)95073-8

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880. https://doi.org/10.1093/oxfordjournals.pcp.a076232

Navarro-León, E., Ruiz, J.M., Graham, N., Blasco, B., 2018. Physiological profile of CAX1a TILLING mutants of *Brassica rapa* exposed to different calcium doses. Plant Sci. 272. https://doi.org/10.1016/j.plantsci.2018.04.019

Noctor, G., Foyer, C.H., 1998. Simultaneous measurement of foliar glutathione, γ-glutamylcysteine, and amino acids by high-performance liquid chromatography: Comparison with two other assay methods for glutathione. Anal. Biochem. 264, 98–110. https://doi.org/10.1006/abio.1998.2794

Nyongesah, M.J., Wang, Q., Li, P., 2015. Effectiveness of photochemical reflectance index to trace vertical and seasonal chlorophyll a/b ratio in *Haloxylon ammodendron*. Acta Physiol. Plant. 37, 2. https://doi.org/10.1007/s11738-014-1747-x

Pittman, J.K., Hirschi, K.D., 2016. CAX-ing a wide net: Cation/H^+ transporters in metal remediation and abiotic stress signaling. Plant Biol. 18, 741–749. https://doi.org/10.1111/plb.12460

Rao, M. V, Paliyath, G., Ormrod, D.P., 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol. 110, 125–36. https://doi.org/https://doi.org/10.1104/pp.110.1.125

Rodríguez-Serrano, M., Romero-Puertas, M.C., Pazmiño, D.M., Testillano, P.S., Risueño, M.C., Del Río, L.A., Sandalio, L.M., 2009. Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. Plant Physiol. 150, 229–43. https://doi.org/10.1104/pp.108.131524

Ryan, P., Delhaize, E., Jones, D., 2001. Function and mechanism of organic anion exudation from plant roots. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 527–560. https://doi.org/10.1146/annurev.arplant.52.1.527

Sandalio, L.M., Rodríguez-Serrano, M., del Río, L.A., Romero-Puertas, M.C., 2009. Reactive Oxygen Species and Signaling in Cadmium Toxicity. Springer, Berlin, Heidelberg, pp. 175–189. https://doi.org/10.1007/978-3-642-00390-5_11

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012, 1–26. https://doi.org/10.1155/2012/217037

Shigaki, T., Barkla, B.J., Miranda-Vergara, M.C., Zhao, J., Pantoja, O., Hirschi, K.D., 2005. Identification of a crucial histidine involved in metal transport activity in the *Arabidopsis* cation/H^+ exchanger CAX1. J. Biol. Chem. 280, 30136–30142. https://doi.org/10.1074/jbc.m503610200

Srere, P.A., 1969. Citric acid cycle, Methods in Enzymology, Methods in Enzymology. Elsevier. https://doi.org/10.1016/0076-6879(69)13005-0

Srivastava, R.K., Pandey, P., Rajpoot, R., Rani, A., Gautam, A., Dubey, R.S., 2015. Exogenous application of calcium and silica alleviates cadmium toxicity by suppressing oxidative damage in rice seedlings. Protoplasma 252, 959–975. https://doi.org/10.1007/s00709-014-0731-z

Strasser, R., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples, in: Yunus, M., Pathre, U., Mohanty, P. (Eds.), Probing Photosynthesis: Mechanism, Regulation and Adaptation. Taylor & Francis, London, pp. 443–480

Sun, R., Zhou, Q., Jin, C., 2006. Cadmium accumulation in relation to organic acids in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccumulator. Plant Soil 285, 125–134. https://doi.org/10.1007/s11104-006-0064-6

Till, B.J., 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res. 13, 524–530. https://doi.org/10.1101/gr.977903

Tran, T.A., Popova, L.P., 2014. Functions and toxicity of cadmium in plants: recent advances and future prospects. Turk. J. Botany 37, 1–13. https://doi.org/10.3906/bot-1112-16

Wang, J., Evangelou, B.P., Nielsen, M.T., Wagner, G.J., 1991. Computer-simulated evaluation of possible mechanisms for quenching heavy metal ion activity in plant vacuoles: I. Cadmium. Plant Physiol. 97, 1154–60. https://doi.org/10.1104/pp.97.3.1154

Willick, I., 2013. Absence of phosphoenolpyruvate carboxylase AtPPC3 increases sensitivity of *Arabidopsis thaliana* to cadmium. Electron. Thesis Diss. Repos.

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Wu, Q., Shigaki, T., Williams, K.A., Han, J.S., Kim, C.K., Hirschi, K.D., Park, S., 2011. Expression of an Arabidopsis Ca^{2+}/H^{+} antiporter CAX1 variant in petunia enhances cadmium tolerance and accumulation. J. Plant Physiol. 168, 167–173. https://doi.org/10.1016/j.jplph.2010.06.005

Yu, Q., Osborne, L., Rengel, Z., 1998. Micronutrient deficiency changes activities of superoxide dismutase and ascorbate peroxidase in tobacco plants. J. Plant Nutr. 21, 1427–1437. https://doi.org/10.1080/01904169809365493

Zhu, Y.-G., Huang, Y.-Z., Hu, Y., Liu, Y.-X., 2003. Iodine uptake by spinach (*Spinacia oleracea* L.) plants grown in solution culture: effects of iodine species and solution concentrations. Environ. Int. 29, 33–37. https://doi.org/10.1016/S0160-4120(02)00129-0

Zorrig, W., Rouached, A., Shahzad, Z., Abdelly, C., Davidian, J.-C., Berthomieu, P., 2010. Identification of three relationships linking cadmium accumulation to cadmium tolerance and zinc and citrate accumulation in lettuce. J. Plant Physiol. 167, 1239–1247. https://doi.org/10.1016/j.jplph.2010.04.012

Supplementary information

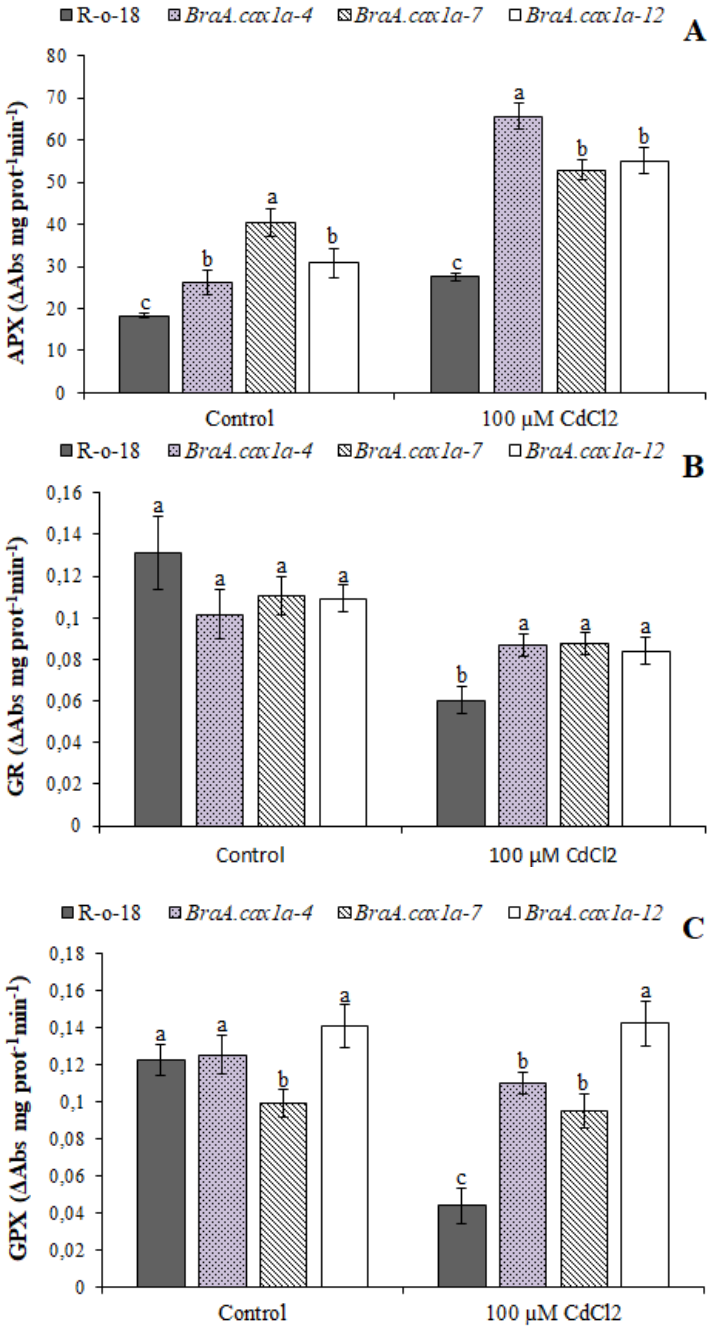

Figure S1. APX (A), GR (B) and GPX (C) activities in *BraA.cax1a* mutants and R-o-18 plants subjected to Cd toxicity. Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

Table S1. Values of oxidative stress indicators and antioxidant enzyme activities in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity

		MDA (μM g^{-1} FW)	LOX (ΔAbs mg $prot^{-1}min^{-1}$)	$O_2^{\bullet-}$ (μg g^{-1} FW)	H_2O_2 (μg g^{-1} FW)	SOD (ΔAbs mg $prot^{-1}min^{-1}$)	CAT (ΔAbs mg $prot^{-1}min^{-1}$)
Control	R-o-18	6.92±0.05c	8.40±1.62c	8.91±0.91b	0.67±0.01a	10.07±0.10c	2.42±0.07b
	BraA.cax1a-4	7.75±0.11b	19.00±1.78a	10.95±0.30a	0.61±0.01b	10.74±0.18b	2.85±0.07a
	BraA.cax1a-7	8.45±0.28a	14.16±1.92b	10.44±0.40a	0.60±0.03b	11.64±0.26a	1.41±0.08d
	BraA.cax1a-12	6.38±0.15d	9.15±1.30b	6.88±0.19c	0.58±0.02b	8.15±0.21d	2.01±0.06c
	p-value	***	***	***	**	***	***
	$LSD_{0.05}$	0.5	4.81	0.78	0.05	0.57	0.21
100 μM $CdCl_2$	R-o-18	10.84±1.59a	24.50±2.31ab	14.71±0.67a	0.77±0.02a	10.67±0.11b	2.08±0.05b
	BraA.cax1a-4	8.72±0.37ab	27.21±1.27a	13.77±0.37a	0.63±0.02b	10.20±0.16b	2.74±0.10a
	BraA.cax1a-7	10.31±0.17a	19.78±1.15b	14.27±0.27a	0.81±0.03a	10.67±0.25b	2.05±0.07b
	BraA.cax1a-12	6.40±0.12b	11.63±2.88c	11.76±0.43b	0.49±0.02c	11.66±0.24a	2.80±0.09a
	p-value	**	***	***	***	***	***
	$LSD_{0.05}$	2.37	6.43	1.32	0.06	0.57	0.22
Analysis of variance							
Cd (C)		***	***	***	***	***	***
Mutation (M)		***	***	***	***	***	***
C x M		*	***	**	***	***	***
$LSD_{0.05}$		1.19	3.72	0.75	0.04	0.40	0.15

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

Table S2. Ascorbate forms concentration and redox state in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity

		Total AsA (μg g^{-1} FW)	AsA (μg g^{-1} FW)	DHA (μg g^{-1} FW)	AsA Redox State
Control	R-o-18	176.71±7.93a	131.02±4.77a	45.69±6.30a	74.21±2.83c
	BraA.cax1a-4	124.04±16.16b	99.00±7.50b	25.04±10.53b	80.48±6.53ab
	BraA.cax1a-7	121.58±11.21b	100.32±4.67b	21.26±8.22b	82.92±5.74a
	BraA.cax1a-12	186.82±29.84a	133.91±26.51a	41.30±9.80a	76.12±5.61bc
	p-value	***	***	***	**
	$LSD_{0.05}$	17.58	13.61	8.51	5.15
100 μM $CdCl_2$	R-o-18	135.56±7.13c	101.96±3.31c	33.60±8.06c	75.41±4.79b
	BraA.cax1a-4	154.97±8.92b	108.07±4.89b	45.90±11.80b	70.46±5.91c
	BraA.cax1a-7	109.12±11.29d	96.15±4.42d	16.59±4.84d	85.42±3.51a
	BraA.cax1a-12	199.09±13.34a	133.92±2.93a	58.82±7.11a	69.56±2.66c
	p-value	***	***	***	***
	$LSD_{0.05}$	10.02	3.81	8.01	4.22
Analysis of variance					
Cd (C)		*	*	**	**
Mutation (M)		***	***	***	***
C x M		***	***	***	***
$LSD_{0.05}$		9.92	6.93	5.73	3.27

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

Table S3. Glutathione forms concentration and redox state in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity

		Total GSH (µg g^{-1} FW)	GSH (µg g^{-1} FW)	GSSG (µg g^{-1} FW)	GSH Redox State
Control	R-o-18	218.39±30.07 a	157.69±43.13 a	60.70±19.35 a	71.19±11.98a b
	BraA.cax1a-4	162.45±23.82 b	99.58±27.98b	62.87±7.76a	60.338.91±c
	BraA.cax1a-7	177.72±27.34 b	112.74±34.49 b	64.98±17.80 a	62.51±12.69b c
	BraA.cax1a-12	225.05±42.11 a	169.62±47.88 a	55.43±11.40 a	74.36±7.67a
	p-value	***	**	NS	*
	$LSD_{0.05}$	30.34	37.57	14.25	10.10
100 µM $CdCl_2$	R-o-18	176.77±35.00 b	119.98±17.95 b	56.79±10.00 b	66.75±3.99b
	BraA.cax1a-4	217.87±17.18 a	146.52±16.17 a	72.35±14.35 a	69.89±5.64ab
	BraA.cax1a-7	215.75±28.30 a	154.40±31.65 a	61.34±8.89b	71.01±6.41ab
	BraA.cax1a-12	213.06±22.03 a	160.02±18.56 a	53.04±5.03b	73.62±1.72a
	p-value	**	**	**	*
	$LSD_{0.05}$	25.44	25.56	9.73	4.36
Analysis of variance					
Cd (C)		*	*	NS	*
Mutation (M)		NS	*	*	*
C x M		***	***	NS	*
$LSD_{0.05}$		21.58	22.29	8.46	5.42

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

CAPÍTULO 3: TOLERANCIA FRENTE A LA SALINIDAD DE TRES MUTANTES CAX1A TILLING DE *Brassica rapa*

3.1. Estudio de la tolerancia al estrés salino y sus mecanismos defensivos en mutantes TILLING CAX1a de *Brassica rapa*

Eloy Navarro-León[1*], Francisco Javier López-Moreno[2], de la Torre-González Alejandro[1], Juan Manuel Ruiz[1], Sergio Esposito[3], y Begoña Blasco[1]

Resumen

La producción de los cultivos se enfrenta al aumento de la salinidad de los suelos y las aguas de riego. Se trata de un problema generalizado que afecta a cultivos de gran importancia económica y reduce sus rendimientos. La salinidad causa estrés oxidativo en las plantas que induce complejos mecanismos de defensa que implican al sistema antioxidante y a los compuestos osmoprotectores. El Ca^{2+} desempeña un papel fundamental para contrarrestar el estrés salino, y la modificación del transportador vacuolar Ca^{2+} CAX1 podría representar un método potencial para mejorar la tolerancia a la salinidad. Se generaron tres nuevas variantes de CAX1 en *Brassica rapa* (*BraA.cax1a*) utilizando la estrategia TILLING. El objetivo de este trabajo es evaluar la tolerancia de estos mutantes a las

condiciones salinas. Para ello, se cultivaron los mutantes *BraA.cax1a* y la línea parental R-o-18 en macetas bajo condiciones control y de salinidad (150 mM NaCl). Se midieron los parámetros de biomasa, acumulación de iones, concentración de compuestos antioxidantes y actividades de las enzimas antioxidantes, la presencia de la proteína de choque térmico 70 (HSP70) y los niveles de prolina (Pro). Según los resultados, la mutación *BraA.cax1a-4* proporcionó una mejor tolerancia al estrés por salinidad. Así pues, las plantas *BraA.cax1a-4* mostraron una mayor biomasa; una acumulación más alta de Ca^{2+} y K^+ en las hojas, una relación Na^+/K^+ más baja, una eliminación de ROS más eficiente (aumento de las actividades de la superóxido dismutasa y la catalasa) y un mayor estado redox del ascorbato. *BraA.cax1a-4* y *BraA.cax1a-12* mostraron un incremento en el transporte de K^+ a las hojas, lo que podría contribuir a su menor peroxidación lipídica. Por el contrario, *BraA.cax1a-7* mostró una respuesta antioxidante más baja. Además, una mayor presencia de isoformas citosólicas y cloroplásticas HSP70 y niveles de Pro podría contribuir a proteger a estos mutantes de *B. rapa* del estrés salino. Por lo tanto, este estudio identifica un posible genotipo útil que podría utilizarse para mejorar la tolerancia a la salinidad de los cultivos.

Adapted from Environmental and Eperimental Botany,

Study of salt-stress tolerance and defensive mechanisms in *Brassica rapa* CAX1a TILLING mutants

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Abstract

Crop production is facing the increase of salinity in soils and irrigation waters. This is a widespread problem that affects crops with high economic importance and reduces yields. Salinity causes oxidative stress in plants which induce complex defensive mechanisms involving the antioxidant system and osmoprotector compounds. Ca^{2+} plays a pivotal role in counteracting salt stress, and the modification of the Ca^{2+} vacuolar transporter CAX1 could represent a potential method to improve tolerance to salinity. Three new CAX1 variants in *Brassica rapa*

(*BraA.cax1a*) were generated using TILLING strategy. The objective of this work is to evaluate the tolerance of these mutants to saline conditions. For this, *BraA.cax1a* mutants and the parental line R-o-18 were grown in pots under control and saline conditions (150 mM NaCl). Parameters of biomass, ions accumulation, antioxidant compounds concentration and antioxidant enzyme activities, heat shock protein 70 (HSP70) occurrence, and proline (Pro) levels were measured. According to the results, *BraA.cax1a-4* mutation provided a higher tolerance to salinity stress. Thus, *BraA.cax1a-4* plants showed higher biomass; higher Ca^{2+} and K^+ accumulation in the shoot, a lower Na^+/K^+ ratio, an enhanced ROS scavenging (increased superoxide dismutase and catalase activities) and an enhanced ascorbate redox state. *BraA.cax1a-4* and *BraA.cax1a-12* showed higher K^+ transport to the shoot which could contribute to their lower lipid peroxidation. In contrast, *BraA.cax1a-7* showed a lower antioxidant response. Besides, an increased occurrence of cytosolic and chloroplastic HSP70 isoforms and Pro levels could contribute to protect these *B. rapa* mutants from saline stress. Therefore, this study identifies a potential useful genotype that could be used to enhance salt tolerance of crops.

***Keywords*:** Calcium, CAX, HSP70, oxidative stress, proline, salinity

1. Introduction

Salinity in crops occurs when the soil or the crop medium presents high concentrations of soluble salts, such as sodium chloride (NaCl) (Munns and Gilliham, 2015). Currently, salinity levels are increasing in cultured soils and irrigation waters, affecting crop production worldwide. This problem affects about 20% of the irrigated land and is observed especially in arid or semi-arid regions (Negrão *et al.*, 2017). In the modifying climate change scenario, salinity in cultivated soils is expected to worsen in the next years (Acosta-Motos *et al.*, 2017). Furthermore, salinity affects crop species with high economic importance such as plants from the Brassicaceae family (rapeseeds, broccoli or cabbage) (Pavlović *et al.*, 2018). Ion toxicity derived from saline stress causes a reduction in growth and thereby causes yield losses. In addition, the accumulation of Na^+ ions alters plant physiology, osmotic potential, nutritional homeostasis and causes oxidative stress (Acosta-Motos *et al.*, 2017).

One of the main symptoms of saline stress is the occurrence of oxidative stress reflected in ROS accumulation (Acosta-Motos *et al.*, 2017). ROS causes the inhibition of growth due to they attack photosystems and scavenging ROS requires energy that is subtracted from the growth process (Adhikari *et al.*, 2019). The disruption of the photosynthesis process produced under saline conditions leads to an irregular energy flux through photosystems. This, in turn, generates an excess of

energy that usually produces ROS and cause photoinhibition. The toxic $O_2^{\cdot-}$ produced is subsequently converted in H_2O_2 by superoxide dismutase (SOD) (Munns and Gilliham, 2015). Besides, ROS causes the destabilization of membranes because of lipid peroxidation which is reflected in higher levels of malondialdehyde (MDA). Thus, MDA is used as an indicator of lipid peroxidation induced by salt-induced oxidative stress (de Azevedo Neto *et al.*, 2006). In steady-state conditions, ROS generation and scavenging are in equilibrium because the antioxidant systems counteract the low ROS generation. However, under stresses such as salinity, this equilibrium often is disturbed which leads to higher ROS accumulation (Acosta-Motos *et al.*, 2017).

Plants mainly use two mechanisms to cope with salinity: the distribution and compartmentalization of toxic ions (Na^+), and the enhancement of antioxidant defenses against oxidative stress (Acosta-Motos *et al.*, 2017). Thus, plants limit Na^+ accumulation, by transporting it to the shoot, or/and accumulating into vacuoles, to reduce toxic effects (Wan *et al.*, 2017). On the other hand, plants' capability to remove the excess of ROS is one of the main factors that provide stress tolerance (Pokotylo *et al.*, 2012). Plants possess antioxidant enzymatic systems such as SODs that eliminate $O_2^{\cdot-}$, and catalase (CAT) and peroxidases that eliminate H_2O_2. In addition, enzymes of the ascorbate-glutathione (AsA/GSH) cycle such as glutathione

reductase (GR) regenerate non-enzymatic antioxidants (Acosta-Motos *et al.*, 2017). Among the non-enzymatic compounds, ascorbate (AsA) and glutathione (GSH) play a key role because of their antioxidant properties (Sharma et al., 2012). GSH eliminates peroxides and is involved in the activation of genes related to stress-response (Gill and Tujeta, 2010). AsA directly scavenges ROS and indirectly through the AsA/GSH cycle (Hasanuzzaman *et al.*, 2011). Another two defense mechanisms involve heat shock proteins (HSP) and proline (Pro). The most abundant HSPs in cells are HSP70s: these chaperons stabilize other proteins, help cellular mechanisms to check protein quality and regulate their degradation. Under abiotic stress, HSP70s bind to misfolded and truncated proteins protecting cells (Usman *et al.*, 2017). On the other hand, plants accumulate low-molecular-weight organic compounds such as Pro that are involved in osmotic regulation. Hence, Pro accumulation is an important indicator of salinity tolerance in plants (Gill and Tujeta, 2010).

Calcium (Ca^{2+}) plays a key role in plant physiology being involved in the structure of cell membranes, in osmotic homeostasis and is a fundamental component of signaling cascades. The involvement of Ca^{2+} in salinity tolerance has been extensively proved (Köster *et al.*, 2019). Toxic Na^{+} concentrations hamper Ca^{2+} function because of the antagonism between these ions; besides, Ca^{2+} favors K^{+}

uptake over Na^+ (Parida and Das, 2005). Thus, Ca^{2+} homeostasis is crucial in salt tolerance: in response to stress, Ca^{2+} fluxes act as a signal activating genes and proteins involved in tolerance mechanisms (Park *et al.*, 2016). Cation exchangers (CAX) transporters are involved in the regulation of Ca^{2+} fluxes in cells (Pokotylo *et al.*, 2012); interestingly, halophyte species such as *Suaeda salsa* present enhanced CAX activities (Han *et al.*, 2011). Hence, the generation of plants with enhanced CAX activity could be a good approach to provide higher salinity tolerance to crops (Pokotylo *et al.*, 2012). CAX1 is the transporter located on vacuole membranes that shows the highest Ca^{2+} transport activity. Three new variants of CAX1 were generated in *Brassica* rapa BraA.CAX1a transporter using TILLING (Targeting Induced Local Lesions in Genomes) (Graham et al., 2014). The objective of this work is to evaluate the tolerance of these mutants to saline conditions. Thus, the hypothesis to test in this study is that these *BraA.CAX1a* variants provide different tolerance to salinity stress and will affect ROS generation and antioxidant responses.

2. Material and methods

2.1. Plant material and growth conditions

B. rapa ssp. trilocularis 'R-o-18' mutants (*BraA.CAX1a*: *BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and

BraA.cax1a-12 (P-to-S change at amino acid 56) and the parent line (R-o-18) were used as plant material for this experiment. Mutant plants were generated as described by Lochlainn *et al.* (2011) and Graham *et al.* (2014). Seeds were germinated on filter paper in Petri dishes and then transferred to pots filled with vermiculite in a growth chamber. The environmental controlled conditions were: humidity (60-80%), temperature (22/18ºC; day/night), photoperiod (14/10-h), and PAR (350 µmol m^{-2} s^{-1}). The plants were watered with a growth solution composed of 6 mM KH_2PO_4, 4 mM KNO_3, 4 mM $Ca(NO_3)_2$ • 4 H_2O, 2 mM $MgSO_4$ • 7 H_2O, 1 mM NaH_2PO_4 • 2 H_2O, 10 µM H_3BO_3, 5 µM Fe-chelate (Sequestrene; 138FeG100), 2 µM $MnCl_2$ • 4 H_2O, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4$ • 5 H_2O, and 0.1 µM Na_2MoO_4 • 2 H_2O. This solution had a pH of 5.5–6.0.

2.2. Experimental design and treatments

Treatments were applied 30 days after germination and were maintained for 21 days. The choice of these times was based on the results obtained in previous cultures with *Brassica rapa* plants grown under salinity and carried out in our research group (data not shown). Plants received two treatments: Control (without NaCl) and salinity (150 mM NaCl). The two factors used in the experiment were salinity (S) and the mutant employed (M). The experimental design comprised a

randomized complete block with eight plants per treatment and three replications each.

2.3. Plant sampling

Plant leaves were rinsed using distilled water, dried on filter paper, and weighed to obtain the fresh weight (FW). A part of all the sample material was frozen at −30ºC to employ it for biochemical assays and another part was lyophilized to determine the dry weight (DW) and the mineral ions concentrations. Total DW was calculated by the sum of leaf and root DW and salt tolerance index (STI) was calculated using the following formula (Mann *et al.*, 2019):

STI = (Total DW of salinity plants / Total DW of control) X 100

2.4. Analysis of Ca^{2+}, Na^{+}, and K^{+} concentrations and selectivity parameters

Ca^{2+}, Na^{+}, and K^{+} concentrations were determined in a sample of 150 mg of lyophilized material subjected to mineralization by wet digestion (Wolf, 1982). The plant material was mineralized using 5 ml of nitric acid (HNO_3)/perchloric acid ($HClO_4$) (v/v) and H_2O_2 at 30%. At the end of the mineralization process, 20 ml of mili-Q H_2O were added and mineral element concentrations were measured using an ICP-MS system (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA).

K+/Na+ selectivity indexes were calculated as (Flowers and Yeo, 1988):

Absorption selectivity (ASK, Na) = ([K+]/[Na+]) in root/ ([K+]/[Na+]) in external solution

Translocation selectivity ($TS_{K, Na}$) = ($[K^+]/[Na^+]$) in roots/ ($[K^+]/[Na^+]$) in leaves.

2.5. ROS concentrations, lipid peroxidation, and antioxidant enzyme activities

The SOD activity was assayed according to the method described by Yu *et al.* (1998) through the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The CAT activity was determined according to the method of Nakano and Asada (1981) measuring the decline of H_2O_2 at 240 nm. The $O_2^{\cdot -}$ determination was performed according to the method of Kubiś (2008) measuring the reduction of NBT at 580 nm. H_2O_2 concentration was colorimetrically measured as described by Junglee *et al.* (2014) at 350 nm. MDA concentration was determined according to Fu and Huang (2001) determining absorbance at 532 nm and correcting turbidity at 600 nm.

Electrolyte leakage (EC) was determined by the electrolyte loss test (Soloklui *et al.*, 2012). For this purpose, 0.3 g of fresh plant material was weighed, lightly washed with deionized water and placed in a test tube, adding 30 mL of deionized

water and the tubes were agitated in a vortex for 1 min. Using a conductivity meter (Cond 8; XS Instruments, Italy), the initial conductivity (EC_1) was measured. The samples were then incubated at 100°C for 20 min to extract the released electrolytes and were cooled to room temperature. Subsequently, the final conductivity (EC_2) was measured. The percentage of electrolyte leakage was obtained using the following formula: (EC_1/EC_2) x 100.

2.6. Lipoxygenase (LOX), peroxidases and GR activities

LOX enzyme activity was recorded according to Minguez-Mosquera *et al.* (1993) using linoleic acid and measuring the absorbance at 234 nm. Ascorbate peroxidase (APX) and GR activities were measured according to Rao *et al.* (1996). To determine APX activity the change of absorbance was registered at 290 nm. GR activity was determined by measuring NADPH oxidation at 340 nm. Glutathione peroxidase (GPX) activity was registered as described by Elia *et al.* (2003) with modifications using H_2O_2 as a substrate and measuring the NADPH oxidation at 340 nm. The protein concentration of extracts was determined according to the Bradford (1976) method.

2.7. GSH and AsA determinations

Oxidized GSH (GSSG), and total GSH (reduced GSH + GSSG) were analyzed according to the method of Noctor and Foyer (1998). This method is based on DTNB reduction and the absorbance is recorded at 412 nm. Reduced GSH levels were obtained as the difference between total GSH and GSSG. Total AsA and reduced AsA concentration were measured using the method of Law *et al.* (1983). This method is based on the reduction of Fe^{3+} by AsA in acid solution and measuring absorbance at 525 nm. The dehydroascorbate (DHA) concentration was obtained from the difference between total AsA and reduced AsA. Redox states of AsA and GSH were obtained using the formula: [(Reduced form) X 100]/ [Reduced + Oxidized forms].

2.8. Immunoblottings

In immunoblottings, proteins were extracted and separated by SDS-PAGE according to the methodology described by Castiglia *et al.* (2015). Then, polypeptides were transferred onto a Hybond nitrocellulose membrane (GE Healthcare). The membrane was incubated with primary antibodies (Agrisera) that bind specifically to HSP70 (Cytosolic, chloroplastic and mitochondrial) and tubulin. After incubation of the membrane with secondary antibodies, the cross-reacting

polypeptides were identified by enhanced chemiluminescence (WesternBrightTM Quantum kit—Advansta, San Josè, CA, USA). The images were acquired by the BioRad Chemidoc system (Bio-Rad, Hercules CA, USA). A densitometric analysis was carried out using Image J (NIH, USA).

2.9. Pro determination

The method of Bieleski and Turner (1966) was used with some modifications for Pro extraction. 0.1 g of fresh leaves were homogenized in 1 ml of MCW (methanol: chloroform: water, 12:5:1). 50 µl of L-2 aminobutyric acid was added as an internal standard. The mixture was centrifuged at 2,300 × *g* for 10 min. The resulting supernatant was added to 700 µl of Milli-Q water and 1.2 ml of chloroform and incubated 24 h at 4 °C. Then, the aqueous phase was obtained, which was lyophilized and the resulting extract was diluted with 0.1 M HCl. Instrumental analysis of soluble AAs was carried out using the precolumn AccQ Tag Ultra Derivatization Kit (Waters, Milford, MA, USA). LC fluorescence analysis was performed on the Waters Acquity® UPLC System equipped with the Acquity fluorescence detector. UPLC separation was performed on the AccQ Tag Ultra column (2.1 x 100 mm, 1.7 µm) from Waters. The flow rate was 0.7 mL min^{-1}, and the column temperature was kept at 55ºC. The injection volume was 1 µL, and the detection was set at a 266-nm excitation wavelength and a 473-nm emission wavelength. The solvent system

consisted of two eluents: 1:20 Dilution of AccQ Tag Ultra eluent A concentrate and AccQ Tag Ultra eluent B.

2.10. Statistical analysis

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics Centurion XVI software. A two-tailed ANOVA was used to determine whether the saline treatment (S), the mutations (M), or the interaction (S * M) significantly affected the results. Means were compared by Fisher's least significant differences (LSD). The significance levels for both analyses were expressed as * P<0.05, ** P<0.01, *** P<0.001, or NS (not significant).

3. RESULTS

3.1. Biomass and STI

The different genotypes used in the experiment did not show different leaf DW when were grown under control conditions. The application of 150 mM NaCl reduced both leaf and root DW in all lines. However, leaf biomass reduction was lower in *BraA.cax1a* plants, especially in *BraA.cax1a-4* plants that showed higher biomass in comparison to other lines both in DW and as in percentage. Likewise, *BraA.cax1a-4* presented higher STI than the rest of the plants. Under control conditions, root DW

of *BraA.cax1a-7* plants was lower, whereas no significant differences were observed when lines were grown under salinity. Nevertheless, *BraA.cax1a-4* and *BraA.cax1a-12* plants registered a higher percentage of reduction in comparison to control conditions (Table 1).

Table 1. Leaf, root biomass, and STI in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		Leaf DW (g plant^{-1})	Root DW (g plant^{-1})	STI (%)
Control	R-o-18	1.93a	0.31ab	
	BraA.cax1a-4	2.00a	0.34a	
	BraA.cax1a-7	1.41a	0.24c	
	BraA.cax1a-12	1.72a	0.28bc	
	p-value	NS	**	
	$LSD_{0.05}$	0.47	0.05	
150 mM NaCl	R-o-18	0.52b (-73%)	0.19a (-39%)	31.93b
	BraA.cax1a-4	0.74a (-63%)	0.18a (-47%)	40.88a
	BraA.cax1a-7	0.49b (-65%)	0.15a (-38%)	37.86b
	BraA.cax1a-12	0.58b (-66%)	0.16a (-43%)	37.25b
	p-value	**	NS	*
	$LSD_{0.05}$	0.14	0.05	3.01
Analysis of variance				
Salinity (S)		***	***	
Mutation (M)		**	**	
S x M		NS	NS	
$LSD_{0.05}$		0.23	0.03	

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The percentage of reduction or increase of the salinity treatment compared to the control is indicated between brackets. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.2. Ions concentration, Na^+/K^+ ratios, and selectivity indexes

Salinity application reduced Ca^{2+} and K^+ contents and increased Na^+ and thereby Na^+/K^+ ratio (Fig. 1A-D). Compared to controls, the four assessed lines registered a similar decrease in leaf Ca^{2+} content (-80%). Although, *BraA.cax1a-7* plants showed a higher increment of Na^+ (342% compared to 200% in R-o-18 and *BraA.cax1a-4*) and *BraA.cax1a-4* presented a lower reduction in K^+ content (-73% compared to -80% in the other lines). Comparing between lines, *BraA.cax1a-4* showed higher Ca^{2+} content but its content was lower in *BraA.cax1a-7* plants grown under saline conditions (Fig. 1A). However, no differences were found in Na^+ content in all lines (Fig. 1B). In addition, *BraA.cax1a-12* and specially *BraA.cax1a-4* showed higher leaf K^+ content in comparison to the other lines (Fig. 1C). These results agree with the lower Na^+/K^+ observed in *BraA.cax1a-4* plants (Fig. 1D).

In roots we observed a different tendency, *BraA.cax1a-4* and *BraA.cax1a-12* showed higher increment in Na^+ levels (more than 600% compared to 540% of R-o-18), and *BraA.cax1a-7* and R-o-18 presented the lowest Na^+ contents (Fig. 1E). All lines presented a similar decrease in root K^+ content compared to control conditions (-60%), although *BraA.cax1a-4* registered the lowest K^+ content in roots, followed by *BraA.cax1a-7,* R-o-18, and *BraA.cax1a-12* that presented the higher content (Fig. 1F). Regarding selectivity indexes, *BraA.cax1a-4* registered the lowest percentage

of increment of $AS_{K, Na}$ (400% compared to more than 500% in the rest of lines) and the highest of $AT_{K, Na}$ (65% compared to -34% in R-o-18 plants). Furthermore, under salinity, *BraA.cax1a-7* and *BraA.cax1a-12* showed lower $AS_{K, Na}$ and *BraA.cax1a-12* registered a light increment in $AT_{K, Na}$ (14%) compared with control conditions (Fig. 1G-H).

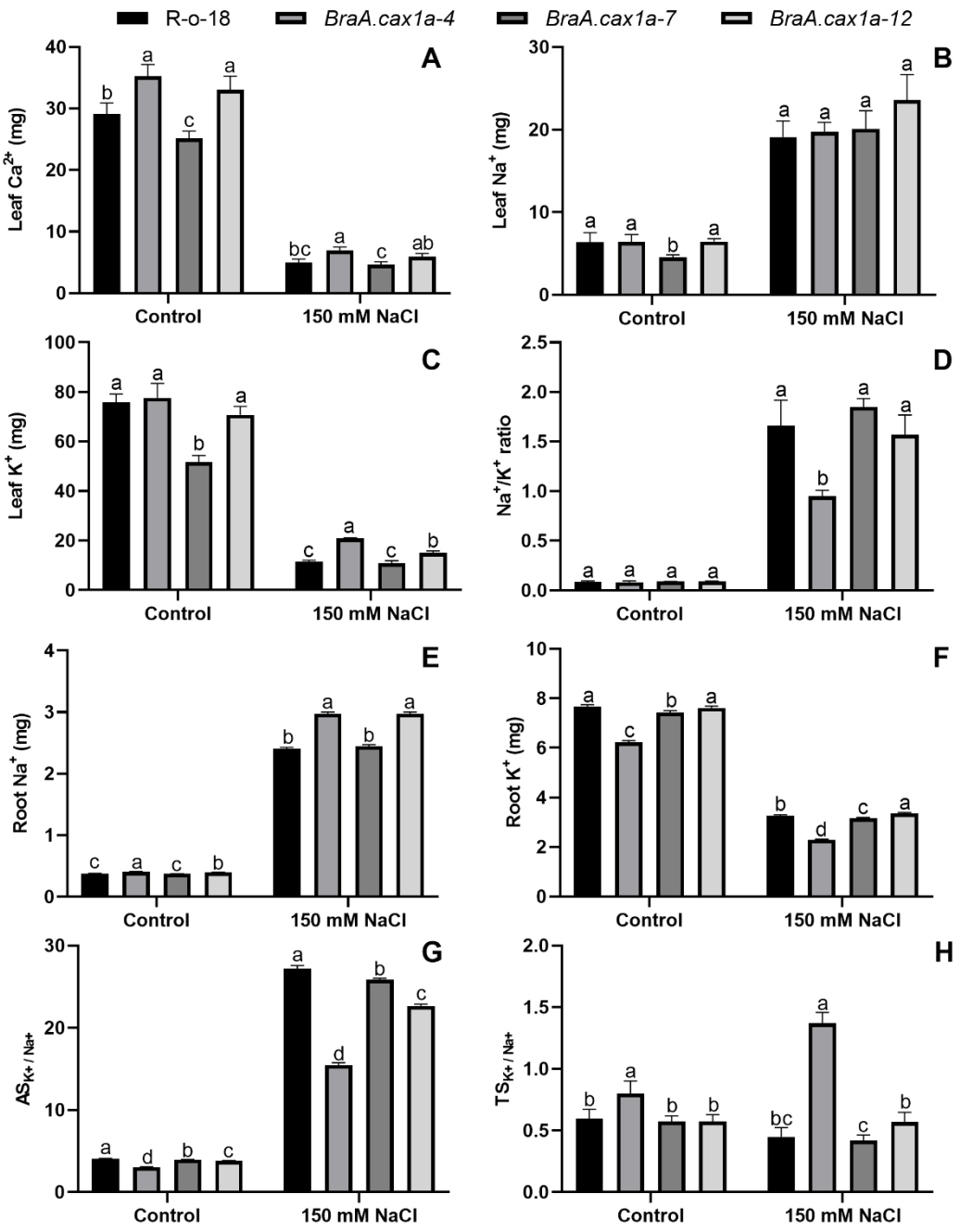

Figure 1. Effect of R-o-18 and *BraA.cax1a* mutations on leaf Ca^{2+} (A), leaf Na^{+} (B), leaf K^{+} (C), Na^{+}/K^{+} ratio (D), root Na^{+} (E), root K^{+} (F), $AS_{K\,/\,Na}$ (G), $TS_{K\,/\,Na}$ (H). Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

3.3. Electrolyte leakage and lipid peroxidation

No different EC and LOX values were found under control conditions in all tested lines. On the other hand, salinity caused an increase in EC, MDA concentration and LOX activity in comparison to controls. However, *BraA.cax1a-4* and *BraA.cax1a-12* plants showed lower EC values than the other lines under salinity. Besides, all *BraA.cax1a* plants registered lower MDA concentration and LOX activity in comparison to R-o-18 plants and *BraA.cax1a-4* registered the lowest values of these two parameters and the lowest percentages of increments for the three analyzed parameters (Table 2).

Table 2. EC percentage, MDA concentration and LOX activity in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		EC	MDA ($\mu M\ g^{-1}$ FW)	LOX ($\Delta Abs\ mg\ prot^{-1} min^{-1}$)
Control	R-o-18	22.50a	5.61b	0.43a
	BraA.cax1a-4	27.03a	6.40a	0.34a
	BraA.cax1a-7	26.52a	6.63a	0.34a
	BraA.cax1a-12	22.60a	4.81c	0.36a
	p-value	NS	***	NS
	$LSD_{0.05}$	7.57	0.71	0.09
150 mM NaCl	R-o-18	54.61a (143%)	11.60a (107%)	0.87a (102%)
	BraA.cax1a-4	41.28c (53%)	6.86c (7%)	0.57c (68%)
	BraA.cax1a-7	50.62ab (91%)	9.42b (42%)	0.69b (103%)
	BraA.cax1a-12	43.34bc (92%)	8.61b (79%)	0.64bc (78%)
	p-value	*	***	***
	$LSD_{0.05}$	8.65	0.85	0.09
Analysis of variance				
Salinity (S)		***	***	***
Mutation (M)		NS	***	***
S x M		*	***	**
$LSD_{0.05}$		5.28	0.54	0.06

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The percentage of reduction or increase of the salinity treatment compared to the control is indicated between brackets. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.4. ROS and antioxidant enzymes

Salinity increased $O_2^{\cdot -}$ concentration in all plants except in *BraA.cax1a-4* that presented similar values compared to control conditions. Salt stress caused a higher accumulation of H_2O_2 in R-o-18 and *BraA.cax1a-7* plants but *BraA.cax1a-4* and *BraA.cax1a-12* showed lower values in comparison to control conditions. All *BraA.cax1a* plants registered lower H_2O_2 values than R-o-18 under saline conditions.

However, the lowest values were observed in *BraA.cax1a-4*, followed by *BraA.cax1a-12*, and *BraA.cax1a-7*. Regarding SOD activity, all plants except *BraA.cax1a-7* increased its activity when NaCl was applied presenting *BraA.cax1a-4* the highest increment percentage compared to control conditions (78%). Thus, *BraA.cax1a-4* showed the highest SOD activity, *BraA.cax1a-7* registered the lowest value and *BraA.cax1a-12* did not present differences in comparison to R-o-18. Concerning CAT activity, salinity decreased its activity in all the plants analyzed. However, *BraA.cax1a-4* plants registered higher CAT activity in comparison to the other lines and the lower reduction percentage compared to control conditions (Table 3).

Table 3. ROS ($O_2^{\bullet-}$ and H_2O_2) concentration and SOD and CAT activities in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		$O_2^{\bullet-}$ (µg g^{-1} FW)	H_2O_2 (µg g^{-1} FW)	SOD (ΔAbs mg $prot^{-1}min^{-1}$)	CAT (ΔAbs mg $prot^{-1}min^{-1}$)
Control	R-o-18	3.22b	189.41b	16.63c	3.52ab
	BraA.cax1a-4	3.68a	202.64a	21.67b	2.63c
	BraA.cax1a-7	3.07b	124.46c	27.98a	2.90bc
	BraA.cax1a-12	3.11b	202.78a	17.24c	3.70a
	p-value	**	***	***	**
	$LSD_{0.05}$	0.33	7.36	2.29	0.62
150 mM NaCl	R-o-18	4.87a (51%)	239.77a (27%)	22.93b (38%)	1.04b (-70%)
	BraA.cax1a-4	3.73b (1%)	160.58d (-21%)	38.47a (78%)	1.74a (-34%)
	BraA.cax1a-7	4.61a (50%)	214.15b (72%)	18.53c (-34%)	1.04b (-64%)
	BraA.cax1a-12	4.66a (50%)	173.76c (-14%)	23.06b (34%)	1.05b (-72%)
	p-value	**	***	***	***
	$LSD_{0.05}$	0.60	10.78	2.71	0.46
Salinity (S)		***	***	***	***
Mutation (M)		NS	***	***	NS
S x M		***	***	***	***
$LSD_{0.05}$		0.34	6.32	1.74	0.33

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The percentage of reduction or increase of the salinity treatment in comparison to the control is indicated between brackets. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.5. AsA/GSH cycle

The application of NaCl increased APX activity in all plants compared to control conditions. *BraA.cax1a-4* plants showed the lowest APX activity and a lesser percentage of increment related to control, whereas the rest of *BraA.cax1a* plants showed similar levels and similar increments in comparison to R-o-18 plants (Fig. 2A). Regarding total AsA, salinity reduced its accumulation in all plants except in *BraA.cax1a-7* plants that presented the highest level and a positive percentage compared to control. Reduced AsA showed a similar tendency as total AsA, but *BraA.cax1a-4* also accumulated more AsA than R-o-18 plants. On the other hand, DHA concentration was lower in *BraA.cax1a-4* and *BraA.cax1a-12* plants but higher in *BraA.cax1a-7* in comparison to R-o-18 plants. In addition, *BraA.cax1a-4* plants showed a 48% reduction in DHA whereas this compound increased in *BraA.cax1a-7* by 201% in comparison to controls. These results produced a greater AsA redox state index in *BraA.cax1a-4*, which showed similar values in the two treatments, followed by *BraA.cax1a-7*, whereas *BraA.cax1a-12* and R-o-18 reached similar levels (Table 4).

Salinity did not induce significant effects on GR and GPX activities in comparison to control conditions. *BraA.cax1a-12* was the only mutant that increased the GR activity, whereas *BraA.cax1a* plants showed a lesser decrease percentage

in comparison to control plants. Under salinity, *BraA.cax1a-4* registered lower GR activity than the other lines, whereas GPX activity was similar between lines except for *BraA.cax1a-7* that showed higher GPX in comparison to *BraA.cax1a-4* plants (Fig. 2B-C). Total GSH concentration and reduced GSH showed a significant decrease in salt-stressed plants, presenting the *BraA.cax1a* plants the lowest levels. Furthermore, *BraA.cax1a-4* plants registered the highest decrease percentage of total GSH and reduced GSH. Nevertheless, *BraA.cax1a-4* was the only line in which GSSG levels remained unchanged, whereas the rest of the lines showed a similar increase. These results lowered GSH redox state index in all *BraA.cax1a* plants and especially in *BraA.cax1a-7* (Table 5).

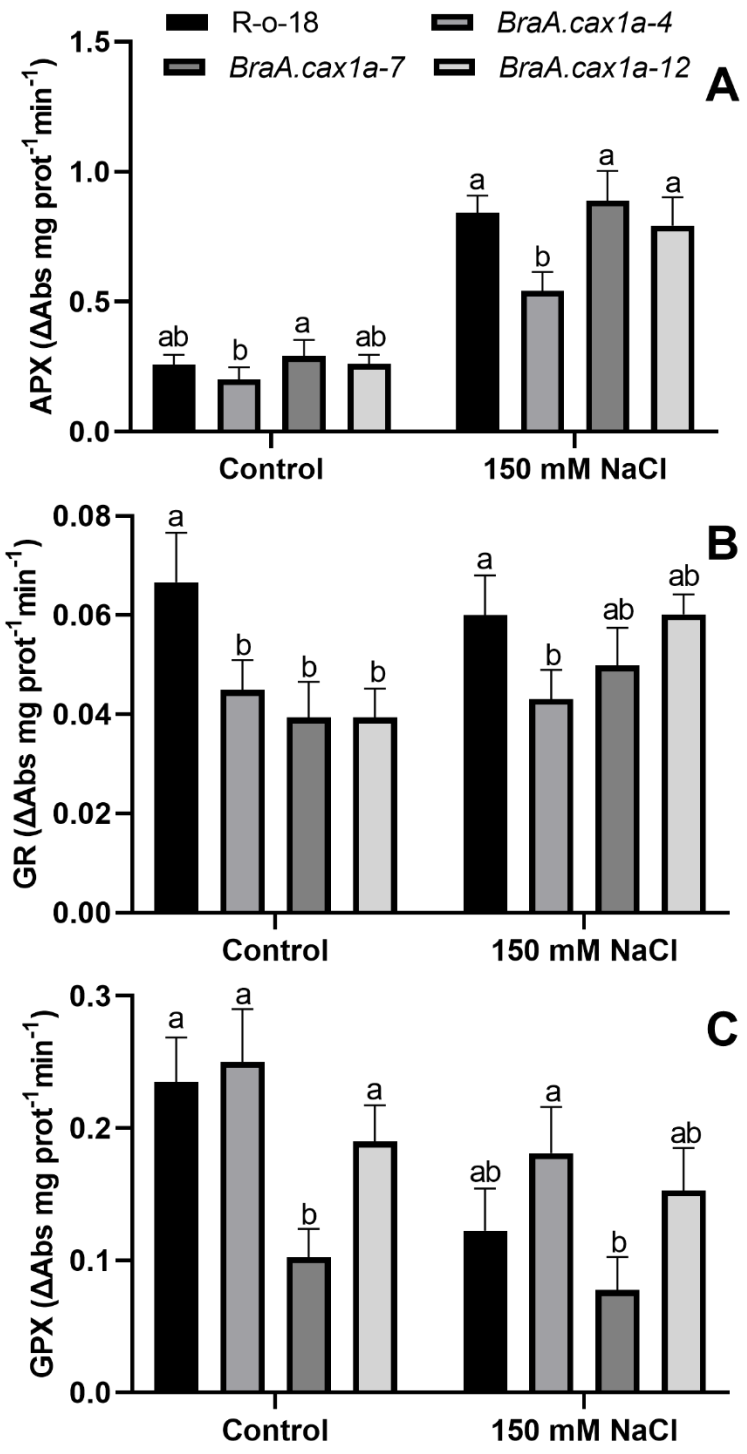

Figure 2. Effect of R-o-18 and *BraA.cax1a* mutations on APX (A), GR (B), and GPX (C) activities. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

Table 4. AsA forms and AsA redox state in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		Total AsA (μg g^{-1} FW)	AsA (μg g^{-1} FW)	DHA (μg g^{-1} FW)	AsA Redox State
Control	R-o-18	105.16a	52.70a	44.44a	54.39ab
	BraA.cax1a-4	94.03a	47.81a	46.22a	50.89b
	BraA.cax1a-7	44.83b	28.04b	16.78b	59.34a
	BraA.cax1a-12	91.29a	50.63a	41.33a	55.47ab
	p-value	***	***	***	*
	$LSD_{0.05}$	14.45	5.68	5.25	5.37
150 mM NaCl	R-o-18	53.91b (-49%)	12.53c (-76%)	42.74b (-4%)	23.44c (-57%)
	BraA.cax1a-4	47.34b (-50%)	22.15b (-54%)	24.02c (-48%)	49.77a (-2%)
	BraA.cax1a-7	82.23a (83%)	31.06a (11%)	50.44a (201%)	37.22b (-37%)
	BraA.cax1a-12	28.70c (-69%)	6.27d (-88%)	25.26c (-39%)	22.81c (-59%)
	p-value	***	***	***	***
	$LSD_{0.05}$	8.10	1.74	5.88	4.15
Analysis of variance					
Salinity (S)		***	***	NS	***
Mutation (M)		***	***	***	***
S x M		***	***	***	***
$LSD_{0.05}$		5.08	2.91	1.37	1.18

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The percentage of reduction or increase of the salinity treatment compared to the control is indicated between brackets. The levels of significance were represented by $p>0.05$: NS (not significant), $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Table 5. GSH forms and GSH redox state in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		Total GSH (µg g^{-1} FW)	GSH (µg g^{-1} FW)	GSSG (µg g^{-1} FW)	GSH Redox State
Control	R-o-18	228.77a	185.22a	43.55c	80.81b
	BraA.cax1a-4	148.54b	107.53b	41.01c	72.21d
	BraA.cax1a-7	118.62c	61.17c	57.45a	51.26c
	BraA.cax1a-12	134.12a	98.12b	51.12b	63.96a
	p-value	***	***	***	***
	$LSD_{0.05}$	14.19	14.00	2.83	3.01
150 mM NaCl	R-o-18	141.86a (-38%)	77.87a (-58%)	57.22ab (31%)	57.22a (-29%)
	BraA.cax1a-4	59.15c (-60%)	23.84c (-78%)	35.32c (-14%)	37.59b (-48%)
	BraA.cax1a-7	66.61c (-44%)	19.77c (-68%)	52.14b (9%)	27.87c (-46%)
	BraA.cax1a-12	97.29b (-27%)	37.01b (-62%)	60.18a (18%)	38.84b (-39%)
	p-value	***	***	***	***
	$LSD_{0.05}$	15.46	9.94	5.82	5.61
Analysis of variance					
Salinity (S)		***	***	***	***
Mutation (M)		***	***	***	***
S x M		***	***	***	**
$LSD_{0.05}$		10.29	8.42	3.96	3.12

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The percentage of reduction or increase of the salinity treatment compared to the control is indicated between brackets. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.6. HSP70 isoforms

NaCl application caused a lower occurrence of Cyt-HSP70 in *BraA.cax1a* plants, whereas a 15% increase was observed in R-o-18 plants in comparison to controls (Fig. 3A). Interestingly, Chl-HSP70 occurrence was increased by 22% only

in *BraA.cax1a-7* plants, whereas in the rest of the lines Chl-HSP70 levels remained substantially unchanged in comparison to controls (Fig. 3B). However, R-o-18 and *BraA.cax1a-4* plants showed a remarkable increase in Mit-HSP70 accumulation under salinity, whereas all the other lines showed a decrease compared to controls (Fig. 3C). The comparison between lines showed that *BraA.cax1a-7* reached the highest accumulations of all HSP70 isoforms. Furthermore, all *BraA.cax1a* plants showed higher levels of Cyt-HSP70 in comparison to R-o-18 and *BraA.cax1a-12* presented the lowest Chl-HSP70 and Mit-HSP70 accumulations (Fig. 3).

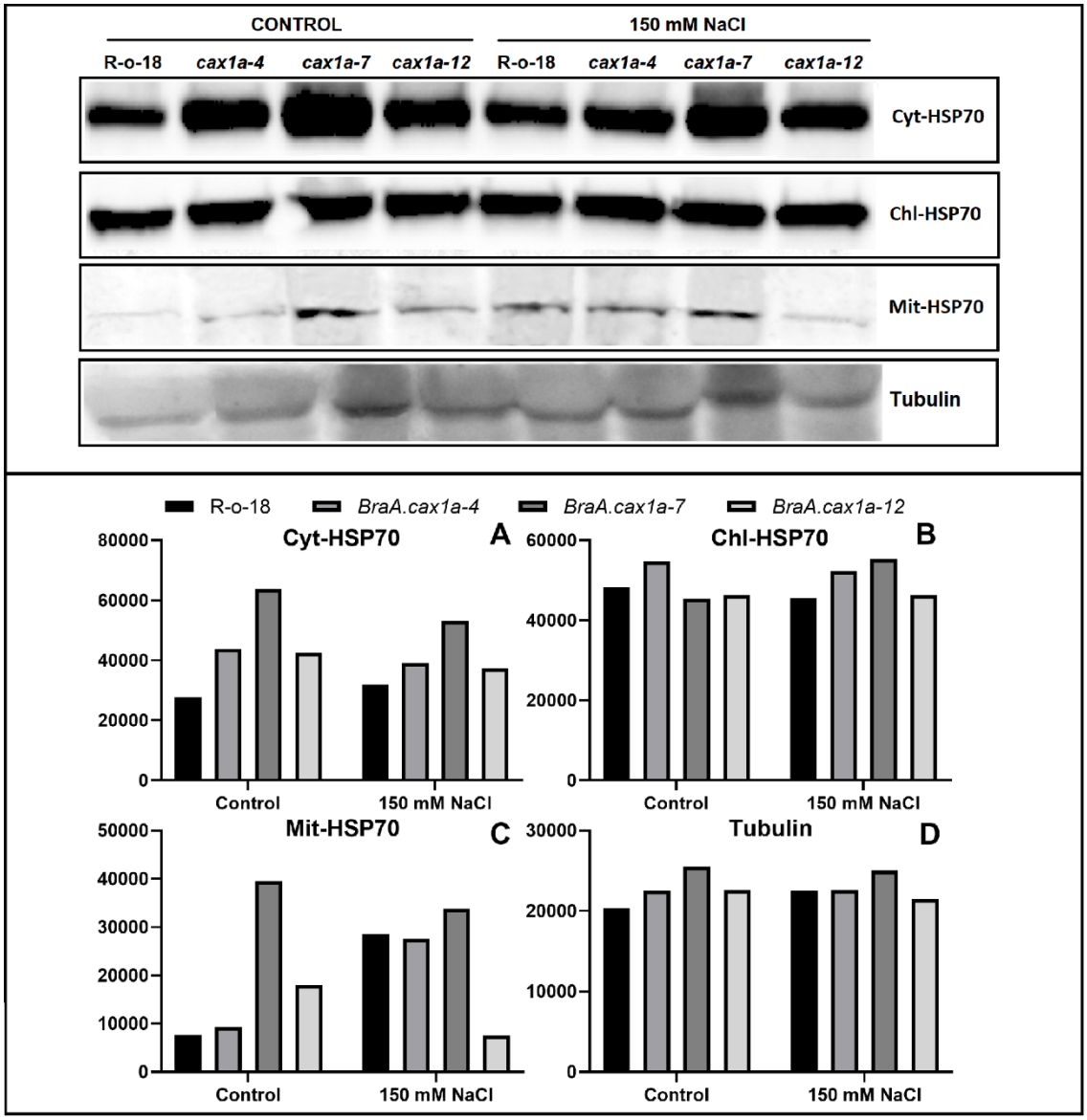

Figure 3. Effect of R-o-18 and *BraA.cax1a* mutations on the accumulation of HSP70. Control blot was performed using anti-tubulin antisera. The upper panel shows western blotting. Lower panel shows histograms that correspond to the band densities in the gels expressed in arbitrary units and calculated by densitometric analysis using Image J.

3.7. Proline concentration

Salinity caused a remarkable increase in Pro accumulation in *B. rapa* plants. Thus, *BraA.cax1a-4* showed the highest increment (over 50-fold) and the highest Pro levels under salinity. In addition, *BraA.cax1a-7* registered a lower Pro concentration, whereas *BraA.cax1a-12* presented similar Pro levels in comparison to R-o-18 plants (Fig. 4).

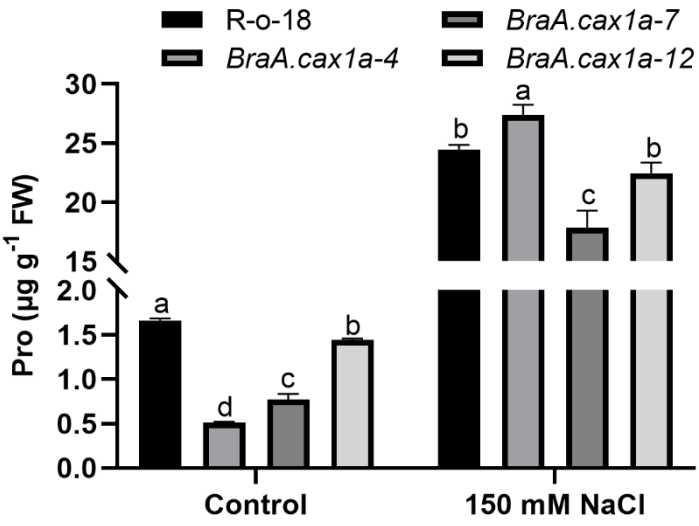

Figure 4. Effect of R-o-18 and *BraA.cax1a* mutations on proline concentration. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

4. Discussion

4.1. Biomass and STI

Salinity causes a reduction in growth and thereby leads to lower biomass as observed in several Brassicaceae crops (Pavlović *et al*., 2018; Sahin *et al*., 2018). In the present study, the biomass was reduced by roughly 70% in shoots and 40% in roots. However, *BraA.cax1a* plants presented lower leaf biomass loss, highlighting *BraA.cax1a-4* plants that presented higher leaf biomass than the other lines which is reflected in a higher STI. García-Martí *et al*. (2019) observed that Ca application increased tolerance to NaCl stress. Likewise, a correct function of Ca^{2+} vacuolar transporter CAX1 is necessary for salt tolerance (Pokotylo *et al.*, 2012). Therefore, the changes in CAX1 transporter provided by *BraA.cax1a-4* mutation could change Ca^{2+} fluxes and enhance NaCl tolerance.

4.2. Ions concentration, Na^+/K^+ ratios, and selectivity indexes

Saline stress leads to the accumulation of Na^+ ions in plant tissues and this accumulation is negatively correlated with salt tolerance in many plant species. Hence, plants restrict Na^+ accumulation, its transport to the shoot or compartmentalize it (Sahin *et al.*, 2018). Likewise, Wan *et al*. (2017) pointed out that in rapeseed salt effects are highly correlated to K^+ and Ca^{2+} homeostasis. Thus, a

lower Na^+/K^+ ratio is associated with higher salt tolerance in *Brassica* species (Pavlović *et al.*, 2018). In the present study, NaCl application increased Na^+ and decreased K^+ and Ca^{2+} contents in all *B. rapa* lines. However, *BraA.cax1a-4* showed a higher K^+ accumulation and thereby lower Na^+/K^+ ratio, which could contribute to better salt tolerance. Previous studies observed that Ca application enhanced NaCl tolerance through a higher K^+ accumulation and a lower Na^+/K^+ ratio (Bacha *et al*., 2015; García-Martí *et al*., 2019). In our study, a better Na^+/K^+ ratio could also be related to a higher Ca^{2+} content in *BraA.cax1a-4* that could improve salt tolerance. Furthermore, *BraA.cax1a-4* mutation might enhance cytosolic Ca^{2+} concentration which activates stress responses as observed in salt-tolerant maize genotypes (Zhao *et al.*, 2019). In the roots, *BraA.cax1a-4* accumulated fewer K^+, and as well as *BraA.cax1a-12* mutants, accumulated higher Na^+ levels. Surprisingly, *BraA.cax1a-4* and *BraA.cax1a-12* presented lower $AS_{K,\,Na}$ which means that they favor Na^+ uptake over K^+. Although, both lines presented higher $TS_{K,\,Na}$ favoring the transport of K^+ over Na^+ to the shoot and thereby increasing Na^+/K^+ in leaves especially in *BraA.cax1a-4*.

4.3. Electrolyte leakage and lipid peroxidation

MDA concentration and LOX activity are indicators of lipid peroxidation caused by salinity (de la Torre-González *et al*., 2017; Chrysargyris *et al*., 2019). Moreover,

Sahin et al. (2018) proved that EC and MDA increased in salt-stressed cabbage plants, which is indicative of membrane damage. In the present study, *B. rapa* showed increments of these parameters in plants grown under salinity, although *BraA.cax1a* plants presented lower values. Similar studies showed the relationship between Ca^{2+} and the protection of membranes against salinity (Bacha *et al*., 2015; Cantabella *et al*., 2017). Besides, Pokotylo *et al*., (2012) observed a relationship between CAX1 activity and lipid peroxidation as measured by higher MDA levels in cax1 mutants subjected to salinity. In our study, *BraA.cax1a-4* and *BraA.cax1a-12* showed lower EC and *BraA.cax1a-4* registered the lowest MDA and LOX values. The lower membrane peroxidation might be related to the higher K^+ concentration measured in these two mutants because lower K^+ levels usually are linked to membrane damages and K^+ concentration may be recovered by Ca application (Bacha *et al*., 2015; Cantabella *et al*., 2017). Therefore, *BraA.cax1a* mutations appear to counteract the membrane damages caused by salinity in *B. rapa* plants.

4.4. ROS and antioxidant enzymes

Salt stress leads to oxidative stress determined by ROS accumulation. Thus, higher $O_2^{\cdot-}$ and H_2O_2 concentrations are measured in plants grown under salinity (Sahin *et al.*, 2018). ROS usually are accumulated in salt-sensitive genotypes (Acosta-Motos *et al.*, 2017). In the present experiment, a higher ROS accumulation

was observed in the majority of plants. However, *BraA.cax1a-4* plants showed lower $O_2^{\cdot-}$ and all *BraA.cax1a* also showed lower H_2O_2 levels, especially *BraA.cax1a-4* plants. To neutralize ROS, plants activate antioxidant defenses such as SOD and CAT activities. Thus, plant genotypes with higher capacity to detoxify ROS through these activities presented higher tolerance to salt (Park *et al*., 2016; García-Martí *et al*., 2019). Our data show that SOD activity was enhanced, but CAT activity was inhibited by salinity. Similarly, Pavlović *et al*. (2018) showed that in *B. rapa* plants CAT was inhibited by salinity possibly by ROS-induced degradation. The lower ROS levels measured in *BraA.cax1a-4* plants were probably due to higher SOD and CAT activities observed in this mutant. In addition, a higher CAT activity reduces the loss of CO_2 by photorespiration and thereby enhances photosynthesis efficiency (Acosta-Motos *et al.*, 2017). On the other hand, *BraA.cax1a-7* had a reduced SOD activity which might increase its sensitivity to $O_2^{\cdot-}$. These results highlight the importance of CAX1 transporter in the activation of antioxidant enzymes as observed in cax1 tobacco plants that presented lower antioxidant activities (Pokotylo *et al.*, 2012).

4.5. AsA/GSH cycle

AsA/GSH cycle is one of the main systems for recycling antioxidants and to scavenge ROS to maintain the redox state in plant cells. Thus, several studies showed increments in the overall or some components of the AsA/GSH cycle as a

consequence of salinity. Besides, genotypes with enhanced AsA/GSH present higher salinity tolerance (Hasanuzzaman *et al*., 2011; Acosta-Motos *et al*., 2017; Zhao *et al*., 2019). In the present study, APX activity was induced by salinity in *B. rapa* plants but in a lesser extent in *BraA.cax1a-4* plants that showed lower APX activity. APX activation probably increases the H_2O_2 detoxification that is generated by salinity. However, *BraA.cax1a-4* could detoxify H_2O_2 mainly through CAT activity. On the other hand, total AsA was reduced by salinity, except in *BraA.cax1a-7*, which might contribute to eliminating ROS in this mutant despite the lower SOD activity. These results agree with other studies in *Brassica* plants grown under salinity stress, in which the authors observed higher APX activity and lower AsA concentration and they associated this decrease to its use in ROS detoxification (Hasanuzzaman *et al*., 2011; Pavlović *et al*., 2018). Furthermore, the AsA/DHA ratio determines the value of AsA redox state and higher values of this parameter indicate higher availability of the reduced form to detoxify ROS and to use it as a substrate for APX (Gill and Tujeta, 2010). *BraA.cax1a-4* showed lower DHA concentration leading to a better AsA redox state which could contribute to enhancing salt tolerance. Regarding GSH, in our experiment, we did not observe a GSH response because GR activity did not show changes caused by salinity and GPX activity was equal or even lower in comparison to control conditions. *BraA.cax1a-4* showed lower GSH concentration and thereby this compound did not have a significant contribution to its higher salt

tolerance. As a whole, *BraA.cax1a* mutations reduced GSH accumulation, specially *BraA.cax1a-7* that presented the lowest GSH redox state.

4.6. HSP70 isoforms

Heat shock proteins represent an important mechanism of protection against stress. The most important and preeminent HSP is HSP70 that under abiotic stress binds to misfolded or truncated proteins protecting cells (Usman *et al*., 2017; Landi, 2019). HSP70s are key in salt-stress tolerance as silenced mutants for HSP70s showed growth inhibition (Anaraki *et al.*, 2018). Besides, the overexpression of HSP70 gene enhanced salinity tolerance (Augustine *et al*., 2015; Fu *et al*., 2016). Our experiment shows that *BraA.cax1a* mutations affect HSP70s levels. Thus, all *BraA.cax1a* mutations showed higher Cyt-HSP70 accumulation which could be related to different cytosolic Ca^{2+} concentrations induced by *BraA.cax1a* mutations. It highlights *BraA.cax1a-7* mutation that showed the highest levels of the three HSP70 isoforms, and particularly Mit-HSP70, suggesting an improved stress response, although HSP70 did not provide a better salt tolerance to this mutant. Recently it has been observed that specific HSP70 isoforms are involved in the response to salinity in barley plants. Thus, stressed plants accumulated Cyt-HSP70 under stress and it should be underlined that this isoform presents different ABA-responsive elements (Landi *et al.*, 2019). *BraA.cax1a* induced mainly Cyt-HSP70

which could be related to the different cytosolic Ca^{2+} fluxes or with the higher ABA levels in these plants (data not shown). Furthermore, *BraA.cax1a-4* and *BraA.cax1a-7* showed higher Chl-HSP70s levels which could protect the photosynthetic machinery in these plants under stress.

4.7. Pro concentration

Pro usually is accumulated after the onset of saline stress and is a key indicator of the stress response. Hence, Pro accumulation is one of the main indicators of saline stress (Cantabella *et al*., 2017; Chrysargyris *et al*., 2019). Pro acts as an osmoprotectant, preserving cell turgor and also contributing to antioxidant activity, scavenging OH^- and $O_2^{\cdot-}$ (Gill and Tujeta, 2010). Accordingly, we noted a remarkable Pro accumulation in plants exposed to salinity: *BraA.cax1a-4* showed a noticeable increase in Pro accumulation which could contribute to the higher salt tolerance observed in these plants. On the other hand, *BraA.cax1a-7* showed the lowest Pro levels and this mutant presented more indicators of salt stress. Anaraki *et al*. (2018) proved that mutants lacking HSP70 partially compensate for it with an increase in Pro levels that can perform similar functions. Therefore, *BraA.cax1a-7* may compensate for the lower Pro levels with higher HSP70s accumulation.

5. Conclusion

Our results indicate that the different *BraA.cax1a* mutations affect salinity response, ROS generation, and antioxidant responses. Thus, *BraA.cax1a-4* mutation could provide a higher tolerance to salinity stress showed by increased biomass. This higher tolerance could be induced by a higher Ca^{+2} and K^{+} contents in the shoot, an enhanced ROS scavenging through SOD and CAT activities and a better AsA redox state. Besides, a higher accumulation of Cyt and Chl HSP70 and Pro could contribute to protect *B. rapa* from salinity. Therefore, this study identifies a potential useful genotype that could be applied to enhance salt tolerance in crops.

Acknowledgments

We thank Dr. Martin R. Broadley and Dr. Neil Graham from Nottingham University for providing us the seeds employed in this experiment.

Funding

This work was supported by the PAI program (Plan Andaluz de Investigación, Grupo de Investigación AGR282) and by a Grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL [FPU14/01858].

REFERENCES

Acosta-Motos, J., Ortuño, M., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M., Hernandez, J., 2017. Plant Responses to Salt Stress: Adaptive Mechanisms. Agronomy 7, 18. https://doi.org/10.3390/agronomy7010018

Adhikari, N.D., Simko, I., Mou, B., 2019. Phenomic and physiological analysis of salinity effects on lettuce. Sensors 19, 4814. https://doi.org/10.3390/s19214814

Anaraki, Z.E., Tafreshi, S.A.H., Shariati, M., 2018. Transient silencing of heat shock proteins showed remarkable roles for HSP70 during adaptation to stress in plants. Environ. Exp. Bot. 155, 142–157. https://doi.org/10.1016/j.envexpbot.2018.06.031

Augustine, S.M., Narayan, J.A., Syamaladevi, D.P., Appunu, C., Chakravarthi, M., Ravichandran, V., Subramonian, N., 2015. Erianthus arundinaceus HSP70 (EaHSP70) overexpression increases drought and salinity tolerance in sugarcane (*Saccharum* spp. hybrid). Plant Sci. 232, 23–34. https://doi.org/10.1016/j.plantsci.2014.12.012

Bacha, H., Ródenas, R., López-Gómez, E., García-Legaz, M.F., Nieves-Cordones, M., Rivero, R.M., Martínez, V., Botella, M.Á., Rubio, F., 2015. High Ca^{2+} reverts the repression of high-affinity K^+ uptake produced by Na^+ in *Solanum lycopersycum* L. (var. *microtom*) plants. J. Plant Physiol. 180, 72–79. https://doi.org/10.1016/j.jplph.2015.03.014

Bieleski, R.L., Turner, N.A., 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Anal. Biochem. 17, 278–93. https://doi.org/10.1016/0003-2697(66)90206-5

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Cantabella, D., Piqueras, A., Acosta-Motos, J.R., Bernal-Vicente, A., Hernández, J.A., Díaz-Vivancos, P., 2017. Salt-tolerance mechanisms induced in *Stevia rebaudiana* Bertoni: Effects on mineral nutrition, antioxidative metabolism and steviol glycoside content. Plant Physiol. Biochem. 115, 484–496. https://doi.org/10.1016/j.plaphy.2017.04.023

Castiglia, D., Cardi, M., Landi, S., Cafasso, D., Esposito, S., 2015. Expression and characterization of a cytosolic glucose 6 phosphate dehydrogenase isoform from barley (*Hordeum vulgare*) roots. Protein Expr. Purif. 112, 8–14. https://doi.org/10.1016/j.pep.2015.03.016

Chrysargyris, A., Solomou, M., Petropoulos, S.A., Tzortzakis, N., 2019. Physiological and biochemical attributes of *Mentha spicata* when subjected to saline conditions and cation foliar application. J. Plant Physiol. 232, 27–38. https://doi.org/10.1016/j.jplph.2018.10.024

de Azevedo Neto, A.D., Prisco, J.T., Enéas-Filho, J., Abreu, C.E.B. de, Gomes-Filho, E., 2006. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ. Exp. Bot. 56, 87–94. https://doi.org/10.1016/j.envexpbot.2005.01.008

de la Torre-González, A., Navarro-León, E., Albacete, A., Blasco, B., Ruiz, J.M., 2017. Study of phytohormone profile and oxidative metabolism as key process to identification of salinity response in tomato commercial genotypes. J. Plant Physiol. 216. https://doi.org/10.1016/j.jplph.2017.05.016

Elia, A.C., Galarini, R., Taticchi, M.I., Dörr, A.J.M., Mantilacci, L., 2003. Antioxidant responses and bioaccumulation in Ictalurus melas under mercury exposure. Ecotoxicol. Environ. Saf. 55, 162–7. https://doi.org/https://doi.org/10.1016/S0147-6513(02)00123-9

Flowers, J.T., Yeo, A.R., 1988. Ion relations of salt tolerance, in: Baker, D.D., Hall, J.L. (Ed.), Solute Transport in Plant Cells and Tissues. John Wiléy and Sons, Inc., New York, pp. 392–416

Fu, C., Liu, X.X., Yang, W.W., Zhao, C.M., Liu, J., 2016. Enhanced salt tolerance in tomato plants constitutively expressing heat-shock protein in the endoplasmic reticulum. Genet. Mol. Res. 15. https://doi.org/10.4238/gmr.15028301

Fu, J., Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45, 105–114. https://doi.org/10.1016/S0098-8472(00)00084-8

García-Martí, M., Piñero, M.C., García-Sanchez, F., Mestre, T.C., López-Delacalle, M., Martínez, V., Rivero, R.M., 2019. Amelioration of the oxidative stress generated by simple or combined abiotic stress through the K^+ and Ca^{2+} supplementation in tomato plants. Antioxidants 8, 81. https://doi.org/10.3390/antiox8040081

Gill, S.., Tujeta, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of *Brassica* under altered Ca supply identifies *Arabidopsis* Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Han, N., Shao, Q., Bao, H., Wang, B., 2011. Cloning and characterization of a Ca^{2+}/H^+ antiporter from halophyte *Suaeda salsa* L. Plant Mol. Biol. Report. 29, 449–457. https://doi.org/10.1007/s11105-010-0244-7

Hasanuzzaman, M., Hossain, M.A., Fujita, M., 2011. Selenium-induced up-regulation of the antioxidant defense and methylglyoxal detoxification system reduces salinity-induced damage in rapeseed seedlings. Biol. Trace Elem. Res. 143, 1704–1721. https://doi.org/10.1007/s12011-011-8958-4

Köster, P., Wallrad, L., Edel, K.H., Faisal, M., Alatar, A.A., Kudla, J., 2019. The battle of two ions: Ca^{2+} signalling against Na^{+} stress. Plant Biol. 21, 39–48. https://doi.org/10.1111/plb.12704

Kubiś, J., 2008. Exogenous spermidine differentially alters activities of some scavenging system enzymes, H_2O_2 and superoxide radical levels in water-stressed cucumber leaves. J. Plant Physiol. 165, 397–406. https://doi.org/10.1016/j.jplph.2007.02.005

Landi, S., Capasso, G., Ben Azaiez, F.E., Jallouli, S., Ayadi, S., Trifa, Y., Esposito, S., 2019. Different roles of heat shock proteins (70 kDa) during abiotic stresses in barley (*Hordeum vulgare*) genotypes. Plants 8, 248. https://doi.org/10.3390/plants8080248

Law, M.Y., Charles, S.A., Halliwell, B., 1983. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. The effect of hydrogen peroxide and of Paraquat. Biochem. J. 210, 899–903. https://doi.org/10.1042/BJ2100899

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R., 2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

Mann, A., Kaur, G., Kumar, A., Sanwal, S.K., Singh, J., Sharma, P.C., 2019. Legume Research: An International Journal., Legume Research - An International Journal. Agricultural Research Communication Centre

Minguez-Mosquera, M.I., Jaren-Galan, M., Garrido-Fernandez, J., 1993. Lipoxygenase activity during pepper ripening and processing of paprika. Phytochemistry 32, 1103–1108. https://doi.org/10.1016/s0031-9422(00)95073-8

Munns, R., Gilliham, M., 2015. Salinity tolerance of crops - what is the cost? New Phytol. 208, 668–673. https://doi.org/10.1111/nph.13519

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880. https://doi.org/10.1093/oxfordjournals.pcp.a076232

Negrão, S., Schmöckel, S.M., Tester, M., 2017. Evaluating physiological responses of plants to salinity stress. Ann. Bot. 119. https://doi.org/10.1093/aob/mcw191

Noctor, G., Foyer, C.H., 1998. Simultaneous measurement of foliar glutathione, γ-glutamylcysteine, and amino acids by high-performance liquid chromatography: Comparison with two other assay methods for glutathione. Anal. Biochem. 264, 98–110. https://doi.org/10.1006/abio.1998.2794

Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60, 324–349. https://doi.org/10.1016/j.ecoenv.2004.06.010

Park, H.J., Kim, W.-Y., Yun, D.-J., 2016. A new insight of salt stress signaling in plant. Mol. Cells 39, 447–59. https://doi.org/10.14348/molcells.2016.0083

Pavlović, I., Pěnčík, A., Novák, O., Vujčić, V., Radić Brkanac, S., Lepeduš, H., Strnad, M., Salopek-Sondi, B., 2018. Short-term salt stress in *Brassica rapa* seedlings causes alterations in auxin metabolism. Plant Physiol. Biochem. 125, 74–84. https://doi.org/10.1016/j.plaphy.2018.01.026

Pokotylo, I. V., Kretinin, S. V., Kravets, V.S., 2012. Role of phospholipase D in metabolic reactions of transgenic tobacco cax1 cells under the influence of salt stress. Cytol. Genet. 46, 131–135. https://doi.org/10.3103/S0095452712030085

Rao, M. V, Paliyath, G., Ormrod, D.P., 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol. 110, 125–36. https://doi.org/https://doi.org/10.1104/pp.110.1.125

Sahin, U., Ekinci, M., Ors, S., Turan, M., Yildiz, S., Yildirim, E., 2018. Effects of individual and combined effects of salinity and drought on physiological, nutritional and biochemical properties of cabbage (*Brassica oleracea* var. capitata). Sci. Hortic. (Amsterdam). 240, 196–204. https://doi.org/10.1016/j.scienta.2018.06.016

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012, 1–26. https://doi.org/10.1155/2012/217037

Soloklui, A.A.G., Ershadi, A., Fallahi, E., 2012. Evaluation of cold hardiness in seven Iranian commercial pomegranate (*Punica granatum* L.) cultivars. HortScience 47(12), 1821–1825. https://doi.org/https://doi.org/10.21273/hortsci.47.12.1821

Usman, M.G., Rafii, M.Y., Martini, M.Y., Yusuff, O.A., Ismail, M.R., Miah, G., 2017. Molecular analysis of Hsp70 mechanisms in plants and their function in response to stress. Biotechnol. Genet. Eng. Rev. 33, 26–39. https://doi.org/10.1080/02648725.2017.1340546

Wan, H., Chen, L., Guo, J., Li, Q., Wen, J., Yi, B., Ma, C., Tu, J., Fu, T., Shen, J., 2017. Genome-wide association study reveals the genetic architecture underlying salt tolerance-related traits in rapeseed (*Brassica napus* L.). Front. Plant Sci. 8, 593. https://doi.org/10.3389/fpls.2017.00593

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Yu, Q., Osborne, L., Rengel, Z., 1998. Micronutrient deficiency changes activities of superoxide dismutase and ascorbate peroxidase in tobacco plants. J. Plant Nutr. 21, 1427–1437. https://doi.org/10.1080/01904169809365493

Zhao, X., Bai, X., Jiang, C., Li, Z., 2019. Phosphoproteomic analysis of two contrasting maize inbred lines provides insights into the mechanism of salt-stress tolerance. Int. J. Mol. Sci. 20, 1886. https://doi.org/10.3390/ijms20081886

3.2. Las mutaciones en CAX1a obtenidas por TILLING modifican el balance hormonal controlando el crecimiento y la homeostasis iónica en plantas de *Brassica rapa* sometidas a salinidad

Eloy Navarro-León, Francisco Javier López-Moreno, Santiago Atero-Calvo, Alfonso Albacete, Juan Manuel Ruiz, and Begoña Blasco

Resumen

La salinidad es un problema grave para los cultivos, ya que causa notables pérdidas de rendimiento. La acumulación de Na^+ afecta a la fisiología de las plantas y produce desequilibrios nutricionales. Las plantas desencadenan cascadas de señales en respuesta al estrés en las que las fitohormonas y el Ca^{2+} son componentes clave. Los antiportadores catión/H^+ (CAX) participan en los flujos de Ca^{2+} en las células. Por lo tanto, una mayor actividad de los CAX podría mejorar la tolerancia al estrés por salinidad. Utilizando la técnica TILLING (targeting induced local lesions in genomes), se generaron tres mutantes de Brassica rapa con una única modificación aminoácida en el transportador CAX1a. Nuestra hipotesis fué que dichas mutaciones podrían modificar el equilibrio hormonal, lo que llevaría a una mejor tolerancia a la salinidad. Para probar esta hipótesis, los mutantes y la línea parental R-o-18 se cultivaron en condiciones de salinidad (150 mM NaCl), y se

analizaron la biomasa de hojas y raíces, las concentraciones de iones y el perfil fitohormonal. En condiciones de salinidad, la mutanción *BraA.cax1a-4* aumentó el crecimiento de las plantas en comparación con la línea parental, lo que se asoció a una menor acumulación de Na^+. Además, aumentó la concentración de K^+ y cambió el equilibrio hormonal. Específicamente, nuestros resultados muestran que las concentraciones más altas de ácido indol-3-acético (IAA) y giberelina (GA) en las plantas mutantes podrían promover el crecimiento en condiciones de salinidad, mientras que el ácido abscísico (ABA), el etileno y el ácido jasmónico (JA) condujeron a una mejor señalización de las respuestas al estrés y a la eficiencia en el uso del agua. Estos resulados demuestran que las mutaciones de CAX1 influyen directamente en el equilibrio hormonal de la planta controlando el crecimiento y la homeostasis iónica en condiciones de salinidad. Por tanto, la manipulación de la señalización de Ca^{2+} puede ser utilizada como una estrategia para mejorar la tolerancia a la salinidad en los programas de breeding.

Adapted from Agronomy 10 (11), 1699.

https://doi.org/10.3390/agronomy10111699

CAX1a TILLING Mutations Modify the Hormonal Balance Controlling Growth and Ion Homeostasis in Brassica rapa Plants Subjected to Salinity

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Abstract

Salinity is a serious issue for crops, as it causes remarkable yield losses. The accumulation of Na^+ affects plant physiology and produces nutrient imbalances. Plants trigger signalling cascades in response to stresses in which phytohormones and Ca^{2+} are key components. Cation/H^+ exchangers (CAXs) transporters are involved in Ca^{2+} fluxes in cells. Thus, enhanced CAX activity could improve tolerance to salinity stress. Using the TILLING (targeting induced local lesions in genomes) technique, three *Brassica rapa* mutants were generated through a single amino acidic modification in the CAX1a transporter. We hypothesized that *BraA.cax1a* mutations could modify the hormonal balance, leading to improved salinity tolerance. To test this hypothesis, the mutants and the parental line R-o-18 were grown under saline conditions (150 mM NaCl), and leaf and root biomass, ion concentrations, and phytohormone profile were analysed. Under saline conditions, *BraA.cax1a-4* mutant plants increased growth compared to the parental line, which was associated with reduced Na^+ accumulation. Further, it increased K^+ concentration and changed the hormonal balance. Specifically, our results show that higher indole-3-acetic acid (IAA) and gibberellin (GA) concentrations in mutant plants could promote growth under saline conditions, while abscisic acid (ABA), ethylene, and jasmonic acid (JA) led to better signalling stress responses and water use efficiency. Therefore, CAX1 mutations directly influence the hormonal balance of the plant controlling growth and

ion homeostasis under salinity. Thus, Ca^{2+} signaling manipulation can be used as a strategy to improve salinity tolerance in breeding programs.

Keywords**:** *Brassica rapa*; calcium; phytohormones; potassium; salinity; sodium

1. Introduction

Saline soils represent 3.1% of the total land area of the Earth. Thereby, salinity is a serious issue for crops because it causes remarkable yield losses (Wu, 2018). This problem has become more important over the last years and it is expected to be even more important in the future because of climate change (Acosta-Motos *et al.*, 2017). Most crop species are affected by salinity including species from the Brassicaceae family, such as cabbage, broccoli, and rapeseed (Pavlović *et al.*, 2018). The most common and soluble salt compound is NaCl. The high concentration of Na^+ and Cl^- ions in saline soils cause osmotic potential imbalances hampering water and nutrients uptake. The accumulation of Na^+ in plants alters the osmotic potential and causes direct toxicity and nutrient imbalances affecting plant physiology (Wu, 2018). The similarity of Na^+ with K^+ hinders K^+ uptake and activity in the plant. Thus, Na^+ accumulates in the cytosol displacing K^+ and also Ca^{2+} ions from their active sites and inhibits enzyme activities. In addition, the altered K^+/Na^+

impairs photosynthesis processes (Köster *et al.*, 2018) and causes oxidative stress, as indicated by a high reactive oxygen species (ROS) accumulation triggering the activation of antioxidant responses (Shoresh *et al.*, 2011; Kravchik and Bernstein, 2013)

As in other stresses, plants trigger phytohormone-mediated responses to cope with saline stress (Acosta-Motos *et al.*, 2017). Plant hormones are compounds from different chemical groups involved in numerous processes in plants. They are crucial for plant adaptation to stress because they mediate adaptive responses that modulate growth, development, and plant nutrition. The resilience of plants to stress is highly dependent on the regulation of hormone signaling pathways (Ku *et al.*, 2018; Sharma *et al.*, 2019). Abscisic acid (ABA) and ethylene have been classically considered stress-related hormones. ABA is an important hormone in salinity response because it regulates the water status via stomata closure and the expression of ABA-responsive genes for long-term responses (Fahad *et al.*, 2015). In addition, ABA regulates the synthesis and accumulation of osmoprotectants, such as proline and some proteins (Iqbal *et al.*, 2014; Pavlović *et al.*, 2018). Alternatively, ethylene synthesis is usually activated as a response to stress and is considered as the main senescence-related hormone. Under salinity, senescence is favored by ABA and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC)

accumulation, as well as by a decrease of indole-3-acetic acid (IAA) and cytokinins (CKs) levels (Acosta-Motos *et al.*, 2017). Indeed, other hormones have been demonstrated to regulate stress responses. Auxins, such as IAA, are involved in the physiological response that modulates oxidative stress and prevents oxidative damage, while CKs protect plants against salinity that maintains growth and delays senescence (Fahad *et al.*, 2015). Other important phytohormones affected by salinity are gibberellins (GAs) associated with sugar signaling and antioxidant system modulation (Khan *et al.*, 2010). Furthermore, jasmonic acid (JA) and salicylic acid (SA) are usually related to biotic stresses, although both hormones are involved in stress signaling in response to salinity and other abiotic stresses (Dar *et al.*, 2015; Fahad *et al.*, 2015).

Besides phytohormones in cooperation with them, Ca^{2+} is a second messenger that fulfills a crucial role in signaling cascades in response to stress (White and Broadley, 2003). During stress signaling, the Ca^{2+} signal is very fast and occurs much earlier than ABA accumulation and, thereby, Ca^{2+} acts in ABA signaling processes (Köster *et al.*, 2018). In response to salinity, Ca^{2+} is involved in salt sensing, Na^{+} extrusion/sequestration, pH regulation, and cellular barriers synthesis (Manishankar *et al.*, 2018). Indeed, the supplementation of plants with $CaCl_2$ has been demonstrated to improve salinity tolerance (Köster *et al.*, 2018). This positive

effect has been observed in many crops including the Brassicaceae species (Latef and Hamed, 2011; Yousuf *et al.*, 2015). Alternatively, seed priming with $CaCl_2$ has beneficial effects on the hormonal balance of the plant alleviating salinity stress symptoms. $CaCl_2$ application reduced ABA and SA levels and increased IAA, promoting plant growth (Iqbal *et al.*, 2006). Besides, Ca^{2+} application maintains membrane integrity, reducing K^+ leakage and preventing Na^+ accumulation, and, thereby, sustaining K^+/Na^+ selectivity (Wu, 2018).

Cation/H^+ exchangers (CAXs) are a family of Ca^{2+}/H^+ antiporters situated on plasma and organelle membranes including vacuoles. CAXs transporters remove Ca^{2+} from the cytosol to generate different Ca^{2+} profiles in the cell. Thus, CAX transporters fulfill a key role in the generation of Ca^{2+} gradients involved in stress signaling (Pittman and Hirschi, 2016). Adequate Ca^{2+} homeostasis driven by CAX and other transporters could be crucial to improving Ca^{2+} fluxes and stress tolerance (Pokotylo *et al.*, 2012), as it was observed in the halophyte species *Suaeda salsa* (Han *et al.*, 2011). Furthermore, CAX1 is the CAX transporter with the highest Ca^{2+}/H^+ activity (Pittman and Hirschi, 2016), thus the modification of CAX1 activity could be useful to improve the tolerance to salinity stress (Pokotylo *et al.*, 2012). TILLING (targeting induced local lesions in genomes) is a promising technique that generates new variants in target genes (Till *et al.*, 2003). Using this technique, three

new variants were produced in *Brassica rapa* ssp. *trilocularis* 'R-o-18′ CAX1a transporter: *BraA.cax1a-4*, *BraA.cax1a-7,* and *BraA.cax1a-12* (Lochlainn *et al.*, 2011). These mutations change amino acids that could affect protein conformation and thereby improve CAX1 function (Graham *et al.*, 2014). As observed in a previous experiment, *BraA.cax1a* mutations induce changes in phytohormone profile (Navarro-León *et al.*, 2019). Given the role of phytohormones in salinity stress, this study aims to test whether changes in Ca^{+2} signaling through *BraA.cax1a* mutations could modify the hormonal balance of the plant leading to improved growth under salinity.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

As plant material, we used three *Brassica rapa* ssp. *trilocularis* 'R-o-18′ mutants (*BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12*) and the parent line R-o-18 (without changes in BraA.CAX1a). The amino acidic changes produced in BraA.CAX1a transporter were: *BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56). Mutant plants were generated as described by Lochlainn *et al*. (2011) and Graham *et al*. (2014). Seeds were germinated on filter paper in Petri

dishes and then transplanted to pots (13 cm × 13 cm × 12.5 cm) filled with vermiculite. Pots were placed in trays (55 cm × 40 cm × 8.5 cm). Plants were grown in a chamber with controlled conditions: relative humidity (60–80%), temperature (23/18 °C; day/night), photoperiod (14/10 h; day/night), and a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} registered at the top of plants using a 190 SB quantum sensor (LI-COR Inc., Lincoln, NE, USA). Plants were supplied with a nutritive solution composed of 6 mM KH_2PO_4, 4 mM KNO_3, 4 mM $Ca(NO_3)_2$•4 H_2O, 2 mM $MgSO_4$•7 H_2O, 1 mM NaH_2PO_4•2 H_2O, 10 µM H_3BO_3, 5 µM Fe-chelate (Sequestrene; 138FeG100), 2 µM $MnCl_2$•4 H_2O, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4$•5 H_2O, and 0.1 µM Na_2MoO_4•2 H_2O. The pH of the nutritive solution was kept between 5.5 and 6.0.

2.2. Experimental Design, Treatments, and Plant Sampling

Treatments were applied 30 days after sowing and were maintained for 21 days. Plants received two different treatments: control (without NaCl added to the nutrient solution) and salinity (150 mM NaCl supplemented to the nutrient solution). The factors considered in the experiment were the salinity (S) and the mutation (M). The experimental design comprised a randomized complete block with 8 treatments, 3 trays per treatment, and 8 plants per tray, thus a total of 24 plants were grown for each treatment. At the end of the experiment, plant leaves and roots were rinsed, dried, and weighed to obtain the fresh weight (FW). Then, leaves and roots were

lyophilized to determine the dry weight (DW) and a part of the lyophilized leaf material was used to determine the phytohormone concentrations. Nine independent replicates from each treatment (*n* = 9) were finally used for the analytical determinations.

2.3. Analysis of Na^+, Ca^{2+}, and K^+ Concentrations

Ca^{2+}, Na^+, and K^+ were determined subjecting the leaf samples to a mineralization process by wet digestion (Wolf, 1982). Next, 150 mg of dry leaves were milled and mineralized with a combination of nitric acid and hydrogen peroxide at 30%. Then, 20 mL of Milli-Q water were added and element concentrations were measured using ICP-MS (X-Series II; Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4. Hormone Extraction and Analysis

Phytohormone concentrations were determined in leaves according to Albacete *et al.* (2008) with some modifications. Lyophilized samples (30 mg) were mixed with 1 mL of cold (−20 °C) extraction mixture of methanol/water (80/20, *v*/*v*). Samples were centrifuged (20,000× *g*, 15 min, 4 °C) and re-extracted for 30 min at 4 °C in 1 mL of the same extraction solution. Supernatants were passed through Sep-Pak Plus †C18 cartridges (SepPak Plus, Waters, Milford, MA 01757 USA) and evaporated at 40 °C under vacuum. The residue was dissolved in 1 mL

methanol/water (20/80, *v*/*v*) using an ultrasonic bath. The dissolved samples were filtered through Millex nylon membrane filters 13 mm diameter of 0.22 µm pore size (Millipore, Bedford, MA, USA). Filtered extracts (10 µL) were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC coupled to an Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a heated electrospray ionization (HESI) interface. The mass spectra were measured using the Xcalibur software version 2.2. For phytohormones quantification, calibration curves were constructed (1, 10, 50, and 100 µg L^{-1}) and corrected for 10 µg L^{-1} deuterated internal standards. Total CKs were calculated as the sum of trans-zeatin (tZ) and isopentenyl adenine (iP) concentrations. Total GAs were calculated as the sum of gibberellin A1 (GA1), gibberellin A3 (GA3), and gibberellin A4 (GA4) concentrations.

2.5. Statistical Analysis

The mean and standard error of each treatment was calculated from the 9 individual data of each parameter analyzed. To assess the differences between treatments we performed a one-way analysis of variance (ANOVA) with 95% confidence. A two-tailed ANOVA was used to determine whether the NaCl treatment (S), the *BraA.cax1a* mutations (M), or their interaction (S × M) significantly influenced the results. Means were compared using Fisher's least significant differences (LSD). The significance levels were stated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, or NS (not significant). A principal components analysis (PCA) was performed to assess

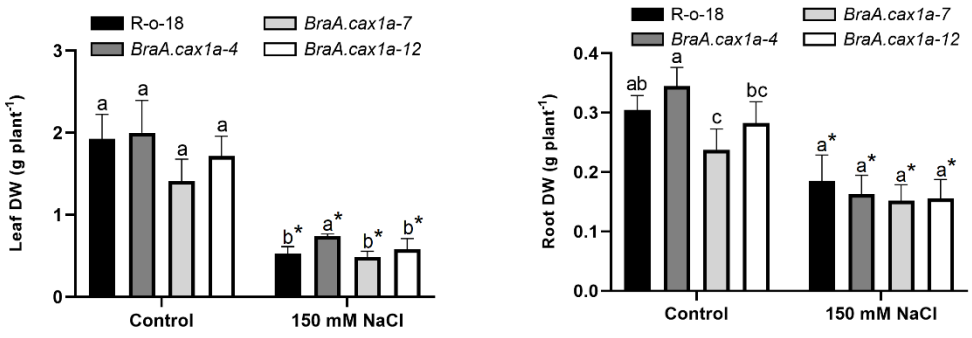
relationships between treatments and all parameters analyzed. All statistical analyses were carried out using the Statgraphics Centurion 16.1.03 software.

3. Results

3.1. Plant Biomass and Cation Concentration

Plants grown under salinity conditions presented a remarkable decrease in leaf and root DW (Figure 1). However, *BraA.cax1a-4* plants grown under salinity showed significantly higher leaf biomass in comparison to the other mutants and the parent line. Indeed, this mutant presented 41% higher leaf DW than R-o-18 plants (Figure 1a). Nonetheless, the four lines analyzed did not show significant differences in root DW under saline conditions (Figure 1b). Regarding cation concentrations, salinity reduced leaf Ca^{2+} concentration in comparison to control conditions in all lines, but no differences were observed between lines under salinity conditions. As expected, NaCl application strongly increased Na^+ concentration in leaves. However, this increment was lower in *BraA.cax1a-4* plants, which showed the lowest Na^+ concentration. Additionally, NaCl application reduced K^+ concentration, although this reduction was lower in *BraA.cax1a-4* plants, which presented the highest K^+ concentration in comparison to the other genotypes. Consequently, *BraA.cax1a-7* plants presented the highest Na^+/K^+ ratio, followed by *BraA.cax1a-12*, R-o-18, and

BraA.cax1a-4 plants. Specifically, *BraA.cax1a-4* mutant plants showed 42% lower Na^+/K^+ ratio than R-o-18 plants (Table 1).



(**a**) (**b**)

Figure 1. Effect of *BraA.cax1a* mutations and salinity on leaf dry weight (DW) (**a**) and root DW (**b**). Values are expressed as means ± standard error (n = 9). Bars marked with different letters indicate significant differences among genotypes based on the LSD test ($p < 0.05$). Asterisk (*) indicates significant differences between control and 150 mM NaCl treatments.

Table 1. Effect of *BraA.cax1a* mutations and salinity on analyzed cation concentration and Na^+/K^+ ratio in leaves.

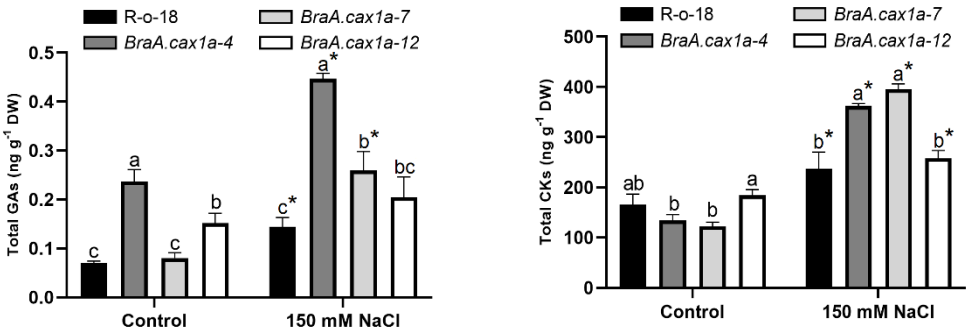
		Ca^{2+}	Na^+	K^+	Na^+/K^+
Control	R-o-18	15.09b	3.29a	39.24ab	0.08a
	BraA.cax1a-4	17.61a	3.64a	38.81ab	0.09a
	BraA.cax1a-7	17.85a	3.23a	36.69b	0.09a
	BraA.cax1a-12	18.51a	3.76a	41.14a	0.09a
	p-value	*	NS	*	NS
	$LSD_{0.05}$	2.22	1.05	4.11	0.02
150 mM NaCl	R-o-18	9.50a	36.74a	22.17b	1.65ab
	BraA.cax1a-4	8.54a	26.65b	28.15a	0.96c
	BraA.cax1a-7	9.57a	41.07a	22.15b	1.85a
	BraA.cax1a-12	10.19a	40.68a	25.85a	1.57b
	p-value	NS	*	**	***
	$LSD_{0.05}$	2.22	9.81	2.55	0.26
	Analysis of variance				
Salinity (S)		***	***	***	***
Mutation (M)		*	*	**	***
S × M		NS	*	*	***
$LSD_{0.05}$		1.44	4.54	2.22	0.12

Values are expressed as mg g−1 DW and differences between means (n = 9) were compared by Fisher's least-significance test (LSD; p = 0.05). Values with different letters indicate significant differences among genotypes. The levels of significance were represented by p > 0.05: NS (not significant), p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).

3.2. Phytohormone Concentrations

Salinity increased total CKs, total GAs, and provoked differential changes in the concentrations of other hormones in R-o-18 and mutant plants (Figure 2; Table 2). Under saline conditions, *BraA.cax1a-4* was the only mutant that showed significantly higher IAA levels (55%) in comparison to R-o-18 plants (Table 2). Regarding GAs under saline conditions, *BraA.cax1a-4* plants presented the highest GA concentrations. The other two mutants presented similar values than R-o-18 plants (Figure 2a). Particularly, *BraA.cax1a-4* showed significant increments in all GAs (4-

fold higher than R-o-18), while in *BraA.cax1a-7* only GA1 increased in comparison to the parent line (Table 2). Concerning CKs, iP decreased in the *BraA.cax1a-4* mutant in comparison to R-o-18, whereas incremented in *BraA.cax1a-7* plants. Importantly, tZ increased in both *BraA.cax1a-4* and *BraA.cax1a-7* mutants, and its absolute concentrations were much higher than those of iP, leading to increased total CK content (Figure 2b). ABA concentration was significantly higher in *BraA.cax1a-4* plants (57%) in comparison to R-o-18 plants. All mutants showed higher ACC levels in comparison to the parent line. SA increased in *BraA.cax1a-4* and *BraA.cax1a-7* mutants, whereas JA concentration increased in *BraA.cax1a-4* and *BraA.cax1a-12* in comparison to the parental R-o-18 (Table 2).



(**a**) (**b**)

Figure 2. Effect of *BraA.cax1a* mutations and salinity on total GA (**a**) and CK (**b**) concentrations in the leaves. Values are expressed as means ± standard error (n = 9). Bars marked with different letters indicate significant differences among genotypes based on the LSD test (p < 0.05). Asterisk (*) indicates significant differences between control and 150 mM NaCl treatments.

Table 2. Effect of *BraA.cax1a* mutations and salinity on leaf phytohormone concentrations

		IAA	GA1	GA3	GA4	iP	tZ	ABA
Control	R-o-18	2.40bc	0.05b	nd	0.02b	6.83a	180.47a	6.43a
	BraA.cax1a-4	3.43b	0.10a	0.08	0.05a	4.55b	129.43ab	4.38ab
	BraA.cax1a-7	1.62c	0.07b	nd	0.01b	2.94b	119.26b	4.13b
	BraA.cax1a-12	5.42a	0.06b	0.05	0.04a	8.54a	176.00a	6.49a
	p-value	**	**		*	***	*	*
	$LSD_{0.05}$	1.55	0.02		0.03	1.29	56.09	2.12
150 mM NaCl	R-o-18	2.03b	0.08c	0.03 c	0.04b	5.10b	231.93b	13.93b
	BraA.cax1a-4	3.14a	0.16ab	0.18 a	0.11a	2.91c	355.02a	21.86a
	BraA.cax1a-7	2.95ab	0.18a	0.05 bc	0.03b	7.82a	382.54a	16.86ab
	BraA.cax1a-12	2.79ab	0.09bc	0.07 b	0.04b	2.94c	255.38b	15.01b
	p-value	*	*	***	**	**	**	*
	$LSD_{0.05}$	1.04	0.07	0.04	0.04	2.05	63.88	5.62
	Analysis of variance							
Salinity (S)		NS	***		**	*	***	***
Mutation (M)		***	***		***	**	NS	NS
S × M		**	NS		NS	***	***	**
$LSD_{0.05}$		0.86	0.03		0.02	1.11	39.07	2.76

Values are expressed as ng g^{-1} DW and differences between means (n = 9) were compared by Fisher's least-signific

different letters indicate significant differences among genotypes. The levels of significance were represented by p > 0

< 0.01 (**), and p < 0.001.

3.3. Principal Component Analysis

A principal component analysis (PCA) was performed to detect general trends in the data and to evaluate the relationships among parameters. The first principal component (PC1) of the score plot clearly separated *BraA.cax1a-4* from the rest of the lines and accounted for 50.55% of the variance within the data. The second principal component (PC2) separated *BraA.cax1a-7* from the other lines and accounted for 23.73% of the variance (Figure 3a). The PCA loading plot revealed three clusters (Figure 3b). The first cluster associated leaf DW with K^+, GA3, GA4, ACC, GAs, and ABA levels. The second cluster related Na^+, Na^+/K^+ ratio, Ca^{2+}, and iP levels. Finally, the third cluster, grouped tZ, total CK, GA1, and SA concentrations (Figure 3b).

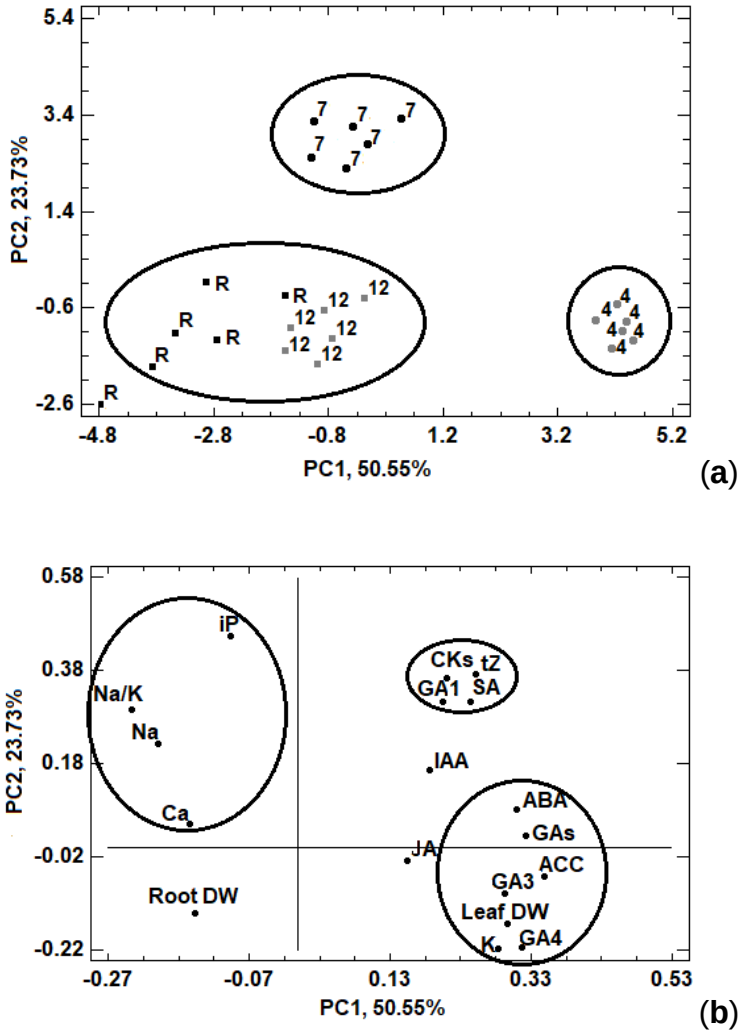


Figure 3. Scores (**a**) and corresponding loadings plot (**b**) of principal component analysis (PCA) using all parameters analyzed in R-o-18 (R), *BraA.cax1a-4* (4), *BraA.cax1a-7* (7), and *BraA.cax1a-12* (12) plants grown under salinity conditions.

4. Discussion

Improved growth responses under salinity are associated with salinity tolerance. According to leaf DW results (Figure 1a), *BraA.cax1a-4* presented higher performance under salinity in comparison to other lines. Several studies observed that a better Ca^{2+} and K^{+} nutrition and homeostasis provide salt tolerance due to Ca^{2+}-associated signaling processes in response to stress and

because it is a Na^+ antagonist (Wan *et al.*, 2017; Köster *et al.*, 2018). In addition, Ca^{2+} improves the accumulation of other nutrients and reduces Na^+/K^+ ratio (White and Broadley, 2003; Khan *et al.*, 2010). Although *BraA.cax1a-4* plants did not present higher Ca^{2+} concentration, they registered lower Na^+ and higher K^+ concentrations, leading to a better Na^+/K^+ ratio (Table 1). A previous study showed that this fact could be due to *BraA.cax1a-4* plants favors the transport of K^+ over Na^+ to the shoot from roots (Navarro-León *et al.*, 2020). Hence, the modification of CAX1 activity could result in different ion accumulation, because of changes in Ca^{2+} fluxes (but not absolute concentrations) in *BraA.cax1a-4,* improving Na^+ and K^+ homeostasis and thereby growth (Mei *et al.*, 2007). This is supported by the close relationship between leaf DW and K^+ concentration observed in loading plot analysis (Figure 3b). Alternatively, Ca^{2+} is involved in modulating ROS levels that are generated by oxidative stress caused by salinity (Shoresh *et al.*, 2011). Thus, as observed in a previous study, the higher tolerance of *BraA.cax1a-4* plants could also be related to this ROS modulation (Navarro-León *et al.*, 2020).

The possible alteration of Ca^{2+} fluxes by *BraA.cax1a* mutations could affect the function of Ca^{2+} sensors, such as calmodulins and protein kinases, that are crucial for hormone synthesis and signaling. Thus, the improved growth response under salinity of *BraA.cax1a-4* plants could be also related to changes in the hormonal balance, as it has been proposed in the present study. Indeed, auxins, and particularly the active compound IAA, have crucial roles in stress signaling responses in *B. rapa* seedlings (Pavlović *et al.*, 2018) and also participate in redox and antioxidative metabolism (Fahad *et al.*, 2015). However, IAA

concentration usually decreases and, thereby, senescence is promoted in plants grown under salt stress (Ghanem *et al.*, 2008). In the present study, we did not observe a significant reduction in IAA concentration. Thus, *BraA.cax1a-4* was the line with the highest IAA concentration, which could contribute to the higher growth observed in this mutant under salinity conditions (Table 2). In fact, the greater IAA levels might enhance ROS detoxification under saline stress which could increase the tolerance to NaCl (de la Torre-González *et al.*, 2017), as previously demonstrated in *BraA.cax1a-4* mutants (Navarro-León *et al.*, 2020).

CKs regulate several physiological processes, promote plant growth, and play important roles in salinity tolerance (Wu *et al.*, 2014; Ma *et al.*, 2016; Pavlů *et al.*, 2018). A decrease in CKs usually is an early response to salt stress (Iqbal *et al.*, 2014), although the contrary was also reported by (Ghanem *et al.*, 2011). These authors observed that CK concentrations increased in plants grown under salt stress as a response to increased salinity tolerance in tomato. This study agrees with our findings as we observed an increment in the CK concentrations in the plants subjected to salinity. Total CK concentrations significantly increased in the mutant, which presented the highest biomass under salinity, *BraA.cax1a-4*, compared to the parent line (Figure 2b). However, their role in the control of growth of *BraA.cax1a* mutants seems to be limited since the PCA analysis revealed that CKs do not associate with any of the growth-related parameters recorded in this assay (Figure 3b).

Some studies have stated that GA accumulation in plants grown under abiotic stress provide salinity tolerance (Park *et al.*, 2016), whereas other studies have shown GA reduction because of repressor protein accumulation, leading to plant growth reduction (Köster *et al.*, 2018). Our results show that total GAs—especially GA3—markedly increased in *BraA.cax1a-4* plants under salinity (Table 2, Figure 2a). GA3 is the main GA that regulates important steps in plant growth and development and alleviates salt stress (Iqbal *et al.*, 2014). Furthermore, Khan *et al.* (2010) proved that the exogenous application of GA3 to linseed, alone or in combination with Ca^{2+}, reduced the damage to membranes and improved water status. Therefore, this particular increment in GA3 and GA4 could also explain the higher biomass of the *BraA.cax1a-4* mutants under salinity stress, as suggested by the close association between GAs and leaf DW in the PCA (Figure 3b).

Despite auxins, CKs, and GAs play a role in the response of the plants to salinity and other abiotic stresses, the phytohormone more broadly studied in relation to water and salinity stress is ABA, since this hormone is over-produced as a consequence of both stresses. Thus, genes related to ABA synthesis and accumulation are up-regulated by NaCl application, and in turn, ABA is the main hormone that activates salt responsive genes (Parida and Das, 2005). Our data reflects an increase in ABA concentration under salinity, which was especially strong in the *BraA.cax1a-4* mutant (Table 2). ABA triggers stress responses, such as water balance and osmotic stress regulation, and leads to stomatal closure to avoid excessive water loss (Zörb *et al.*, 2019). This fact could be important in *BraA.cax1a-4* plants where the high ABA levels provoke the closure of the

stomata, thus enhancing water use efficiency (WUE) (data not shown). This is in agreement with previous studies in maize and tomato in which salinity-tolerant genotypes presented greater WUE and stomatal regulation, and lower Na^+ accumulation than the sensitive ones, associated with higher ABA concentrations (Zörb *et al.*, 2013; Amjad *et al.*, 2014). Furthermore, Iqbal *et al*. (2014) proved that Ca^{2+} induces ABA accumulation, thus reducing Na^+ and Cl^- content and increasing K^+ content. Therefore, ABA could contribute to better ion homeostasis via Na^+/K^+ reduction, as observed in *BraA.cax1a-4* plants (Table 1), maintain growth in plants grown under salinity conditions. This is further supported by the linkage among ABA, K^+, and leaf FW shown in the PCA plot (Figure 3b).

Another hormone traditionally associated with stress responses is ethylene, especially in relation to leaf senescence and abscission. Salinity stress triggers the accumulation of the ethylene precursor ACC, leading to *de-novo* synthesis of ethylene, which induces cell death and leaf senescence, and reduces plant growth (Fahad *et al.*, 2015). In our study, ACC concentration was higher in plants subjected to salinity which could contribute to biomass reduction (Table 2, Figure 1). Likewise, de la Torre-González *et al.* (2017) observed that a salinity-tolerant tomato genotype presented much lower ACC concentration than the sensitive one under saline conditions. However, some studies proved that plants supplied with ACC or that overexpress ethylene-responsive factors presented tolerance to salinity stress (Park *et al.*, 2016; Trivellini *et al.*, 2016). Thus, all mutants evaluated in the present study showed a significant increase in ACC concentrations, and importantly, the *BraA.cax1a-4* mutant presented the highest concentrations (Table 2) which may be associated with its improved growth-

response due to better regulation of Ca^{2+} homeostasis under salinity. This conclusion is additionally endorsed by the link of ACC with leaf FW and K^+ (Figure 3b). Indeed, the increased growth of *BraA.cax1a-4* mutant under salinity stress could be partially associated with improved ethylene regulation of the high-affinity K^+ transporters, which increase K^+ tissue accumulation (Trivellini *et al.*, 2016), as observed in this mutant (Table 1).

SA and JA regulate biotic stress responses but also are involved in regulating abiotic stresses, such as salinity, in cooperation with other hormones (Dar *et al.*, 2015; Arif *et al.*, 2020). JA appears to provide tolerance to salt stress via plant signal transduction (Ku *et al.*, 2018), whereas SA induces antioxidant defenses, protects photosynthesis, and reduces membrane damage (Iqbal *et al.*, 2014; Farhangi-Abriz and Ghassemi-Golezani, 2018). Iqbal *et al.* (2014) observed that JA reduces membrane depolarization and, thereby, K^+ loss. Hence, the highest JA concentrations of *BraA.cax1a-4* and *BraA.cax1a-12* (Table 2) could prevent K^+ leakage and contribute to the higher K^+ concentrations detected in these two mutants (Table 1). Additionally, this also may be explained by the lower lipid peroxidation levels previously observed in *BraA.cax1a-4* and *BraA.cax1a-12* plants (Navarro-León *et al.*, 2020). Regarding SA, we observed a significant increment in SA concentration in all mutant plants grown under salinity in comparison to the parental R-o-18 plants (Table 2). Ku *et al.* (2018) proved that increments in cytosolic Ca^{2+} are a necessary step in SA accumulation in response to salinity. Thus, *BraA.cax1a* mutations could induce a higher SA accumulation through a greater cytosolic Ca^{2+} response.

5. Conclusions

The present study demonstrates that the control of Ca^{2+} homeostasis through *BraA.cax1a* mutants modifies the phytohormone balance of *B. rapa* plants controlling growth responses under salinity stress conditions. Specifically, the growth improvement of *BraA.cax1a-4* mutant plants under salinity can be primarily associated with higher GA, IAA, and CK concentrations. Besides, ABA accumulation activates signaling stress responses under saline conditions, leading to better control of Na^+/K^+ homeostasis. This study confirms the key role of the CAX1 transporter in phytohormone regulation and as a potential target for improving growth under salinity stress. However, further research is still necessary to mechanistically demonstrate the relationship between Ca^{2+} fluxes and phytohormone accumulation in the control of the growth and productivity under salinity conditions.

Funding

This research was funded by the PAI program (Plan Andaluz de Investigación, Grupo de Investigación AGR282) and by a Grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL grant number [FPU14/01858].

Acknowledgments

We thank Martin R. Broadley and Neil Graham from Nottingham University for providing us the seeds utilized in this experiment. We also thank María del Puerto Sánchez-Iglesias for technical support with hormone analyses.

References

Acosta-Motos, J., Ortuño, M., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M., Hernandez, J., 2017. Plant responses to salt stress: Adaptive mechanisms. Agronomy 7, 18. https://doi.org/10.3390/agronomy7010018

Albacete, A., Ghanem, M.E., Martinez-Andujar, C., Acosta, M., Sanchez-Bravo, J., Martinez, V., Lutts, S., Dodd, I.C., Perez-Alfocea, F., 2008. Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (*Solanum lycopersicum* L.) plants. J. Exp. Bot. 59, 4119–4131. https://doi.org/10.1093/jxb/ern251

Amjad, M., Akhtar, J., Anwar-ul-Haq, M., Yang, A., Akhtar, S.S., Jacobsen, S.-E., 2014. Integrating role of ethylene and ABA in tomato plants adaptation to salt stress. Sci. Hortic. (Amsterdam). 172, 109–116. https://doi.org/10.1016/j.scienta.2014.03.024

Arif, Y., Sami, F., Siddiqui, H., Bajguz, A., Hayat, S., 2020. Salicylic acid in relation to other phytohormones in plant: A study towards physiology and signal transduction under challenging environment. Environ. Exp. Bot. 175, 104040. https://doi.org/10.1016/j.envexpbot.2020.104040

Dar, T.A., Uddin, M., Khan, M.M.A., Hakeem, K.R., Jaleel, H., 2015. Jasmonates counter plant stress: A Review. Environ. Exp. Bot. 115, 49–57. https://doi.org/10.1016/j.envexpbot.2015.02.010

de la Torre-González, A., Navarro-León, E., Albacete, A., Blasco, B., Ruiz, J.M., 2017. Study of phytohormone profile and oxidative metabolism as key process to identification of salinity response in tomato commercial genotypes. J. Plant Physiol. 216. https://doi.org/10.1016/j.jplph.2017.05.016

Fahad, S., Hussain, S., Matloob, A., Khan, F.A., Khaliq, A., Saud, S., Hassan, S., Shan, D., Khan, F., Ullah, Najeeb, Faiq, M., Khan, M.R., Tareen, A.K., Khan, A., Ullah, A., Ullah, Nasr, Huang, J., 2015. Phytohormones and plant responses to salinity stress: a review. Plant Growth Regul. 75, 391–404. https://doi.org/10.1007/s10725-014-0013-y

Farhangi-Abriz, S., Ghassemi-Golezani, K., 2018. How can salicylic acid and jasmonic acid mitigate salt toxicity in soybean plants? Ecotoxicol. Environ. Saf. 147, 1010–1016. https://doi.org/10.1016/j.ecoenv.2017.09.070

Ghanem, M.E., Albacete, A., Smigocki, A.C., Frébort, I., Pospíšilová, H., Martínez-Andújar, C., Acosta, M., Sánchez-Bravo, J., Lutts, S., Dodd, I.C., Pérez-Alfocea, F., 2011. Root-synthesized cytokinins improve shoot growth and fruit yield in salinized tomato (*Solanum lycopersicum* L.) plants. J. Exp. Bot. 62, 125–140. https://doi.org/10.1093/jxb/erq266

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of Brassica under altered Ca supply identifies *Arabidopsis* Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Han, N., Shao, Q., Bao, H., Wang, B., 2011. Cloning and characterization of a Ca^{2+}/H^{+} antiporter from halophyte *Suaeda salsa* L. Plant Mol. Biol. Report. 29, 449–457. https://doi.org/10.1007/s11105-010-0244-7

Iqbal, M., Ashraf, M., Jamil, A., Ur-Rehman, S., 2006. Does seed priming induce changes in the levels of some endogenous plant hormones in hexaploid wheat plants under salt stress? J. Integr. Plant Biol. 48, 181–189. https://doi.org/10.1111/j.1744-7909.2006.00181.x

Iqbal, N., Umar, S., Khan, N.A., Khan, M.I.R., 2014. A new perspective of phytohormones in salinity tolerance: Regulation of proline metabolism. Environ. Exp. Bot. 100, 34–42. https://doi.org/10.1016/j.envexpbot.2013.12.006

Khan, M.N., Siddiqui, M.H., Mohammad, F., Naeem, M., Khan, M.M.A., 2010. Calcium chloride and gibberellic acid protect linseed (*Linum usitatissimum* L.) from NaCl stress by inducing antioxidative defence system and osmoprotectant accumulation. Acta Physiol. Plant. 32, 121–132. https://doi.org/10.1007/s11738-009-0387-z

Köster, P., Wallrad, L., Edel, K.H., Faisal, M., Alatar, A.A., Kudla, J., 2018. The battle of two ions: Ca^{2+} signalling against Na^{+} stress. Plant Biol. 21, 39–48. https://doi.org/10.1111/plb.12704

Kravchik, M., Bernstein, N., 2013. Effects of salinity on the transcriptome of growing maize leaf cells point at cell-age specificity in the involvement of the antioxidative response in cell growth restriction. BMC Genomics 14, 24. https://doi.org/10.1186/1471-2164-14-24

Ku, Y.-S., Sintaha, M., Cheung, M.-Y., Lam, H.-M., 2018. Plant hormone signaling crosstalks between biotic and abiotic stress responses. Int. J. Mol. Sci. 19, 3206. https://doi.org/10.3390/ijms19103206

Latef, A., Hamed, A.A., 2011. Ameliorative effect of calcium chloride on growth, antioxidant enzymes, protein patterns and some metabolic activities of canola (*Brassica napus* L.) under seawater stress. J. Plant Nutr. 34, 1303–1320. https://doi.org/10.1080/01904167.2011.580817

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R., 2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

Ma, X., Zhang, J., Huang, B., 2016. Cytokinin-mitigation of salt-induced leaf senescence in perennial ryegrass involving the activation of antioxidant systems and ionic balance. Environ. Exp. Bot. 125, 1–11. https://doi.org/10.1016/j.envexpbot.2016.01.002

Manishankar, P., Wang, N., Köster, P., Alatar, A.A., Kudla, J., 2018. Calcium signaling during salt stress and in the regulation of ion homeostasis. J. Exp. Bot. 69, 4215–4226. https://doi.org/10.1093/jxb/ery201

Mei, H., Zhao, J., Pittman, J.K., Lachmansingh, J., Park, S., Hirschi, K.D., 2007. In planta regulation of the Arabidopsis Ca^{2+}/H^{+} antiporter CAX1. J. Exp. Bot. 58, 3419–3427. https://doi.org/10.1093/jxb/erm190

Navarro-León, E., López-Moreno, F.J., de la Torre-González, A., Ruiz, J.M., Esposito, S., Blasco, B., 2020. Study of salt-stress tolerance and defensive mechanisms in *Brassica rapa* CAX1a TILLING mutants. Environ. Exp. Bot. 175, 104061. https://doi.org/10.1016/j.envexpbot.2020.104061

Navarro-León, E., Ruiz, J.M., Albacete, A., Blasco, B., 2019. Effect of CAX1a TILLING mutations and calcium concentration on some primary metabolism processes in *Brassica rapa* plants. J. Plant Physiol. 237, 51–60. https://doi.org/10.1016/j.jplph.2019.04.002

Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60, 324–349. https://doi.org/10.1016/j.ecoenv.2004.06.010

Park, H.J., Kim, W.-Y., Yun, D.-J., 2016. A new insight of salt stress signaling in plant. Mol. Cells 39, 447–59. https://doi.org/10.14348/molcells.2016.0083

Pavlović, I., Pěnčík, A., Novák, O., Vujčić, V., Radić Brkanac, S., Lepeduš, H., Strnad, M., Salopek-Sondi, B., 2018. Short-term salt stress in *Brassica rapa* seedlings causes alterations in auxin metabolism. Plant Physiol. Biochem. 125, 74–84. https://doi.org/10.1016/j.plaphy.2018.01.026

Pavlů, J., Novák, J., Koukalová, V., Luklová, M., Brzobohatý, B., Černý, M., 2018. Cytokinin at the crossroads of abiotic stress signalling pathways. Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19082450

Pittman, J.K., Hirschi, K.D., 2016. CAX-ing a wide net: Cation/H^+ transporters in metal remediation and abiotic stress signaling. Plant Biol. 18, 741–749. https://doi.org/10.1111/plb.12460

Pokotylo, I. V., Kretinin, S. V., Kravets, V.S., 2012. Role of phospholipase D in metabolic reactions of transgenic tobacco cax1 cells under the influence of salt stress. Cytol. Genet. 46, 131–135. https://doi.org/10.3103/S0095452712030085

Sharma, A., Shahzad, B., Kumar, V., Kohli, S.K., Sidhu, G.P.S., Bali, A.S., Handa, N., Kapoor, D., Bhardwaj, R., Zheng, B., 2019. Phytohormones regulate accumulation of osmolytes under abiotic stress. Biomolecules 9, 285. https://doi.org/10.3390/biom9070285

Shoresh, M., Spivak, M., Bernstein, N., 2011. Involvement of calcium-mediated effects on ROS metabolism in the regulation of growth improvement under salinity. Free Radic. Biol. Med. 51, 1221–1234. https://doi.org/10.1016/j.freeradbiomed.2011.03.036

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N.E., Henikoff, J.G., Comai, L., Henikoff, S., 2003. Large-scale discovery of induced point mutations with high-

throughput TILLING. Genome Res. 13, 524–530. https://doi.org/10.1101/gr.977903

Trivellini, A., Lucchesini, M., Ferrante, A., Carmassi, G., Scatena, G., Vernieri, P., Mensuali-Sodi, A., 2016. Survive or die? A molecular insight into salt-dependant signaling network. Environ. Exp. Bot. 132, 140–153. https://doi.org/10.1016/j.envexpbot.2016.07.007

Wan, H., Chen, L., Guo, J., Li, Q., Wen, J., Yi, B., Ma, C., Tu, J., Fu, T., Shen, J., 2017. Genome-wide association study reveals the genetic architecture underlying salt tolerance-related traits in rapeseed (*Brassica napus* L.). Front. Plant Sci. 8, 593. https://doi.org/10.3389/fpls.2017.00593

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511. https://doi.org/10.1093/aob/mcg164

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Wu, H., 2018. Plant salt tolerance and Na^+ sensing and transport. Crop J. 6, 215–225. https://doi.org/10.1016/j.cj.2018.01.003

Wu, X., He, J., Chen, J., Yang, S., Zha, D., 2014. Alleviation of exogenous 6-benzyladenine on two genotypes of eggplant (*Solanum melongena* Mill.) growth under salt stress. Protoplasma 251, 169–176. https://doi.org/10.1007/s00709-013-0535-6

Yousuf, P.Y., Ahmad, A., Hemant, M., Ganie, A.H., Aref, I.M., Iqbal, M., 2015. Potassium and calcium application ameliorates growth and oxidative homeostasis in salt-stressed indian mustard (*Brassica Juncea*) plants. Pak. J. Bot 47, 1629–1639

Zörb, C., Geilfus, C.-M., Mühling, K.H., Ludwig-Müller, J., 2013. The influence of salt stress on ABA and auxin concentrations in two maize cultivars differing in salt resistance. J. Plant Physiol. 170, 220–224. https://doi.org/10.1016/j.jplph.2012.09.012

Zörb, C., Geilfus, C. -M., Dietz, K. -J., 2019. Salinity and crop yield. Plant Biol. 21, 31–38. https://doi.org/10.1111/plb.12884

3.3. Efecto de mutaciones en CAX1a obtenidas por TILLING sobre el rendimiento fotosintético en plantas de *Brassica rapa* sometidas a estrés salino

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Resumen

La salinidad es un importante factor ambiental que reduce la productividad de los cultivos en muchas regiones del mundo. Afecta negativamente a la fotosíntesis provocando una reducción del crecimiento. Asimismo, el calcio (Ca^{2+}) es crucial en la respuesta de las plantas frente al estrés. Por lo tanto, la modificación de los transportadores de cationes Ca^{2+} (CAX) podría ser una estrategia potencial para aumentar la tolerancia de las plantas a la salinidad. Mediante el uso de Targeting Induced Local Lesions in Genomes (TILLING), los investigadores generaron tres mutantes del transportador CAX1a de *Brassica rapa*: *BraA.cax1a-7*, *BraA.cax1a-4* y *BraA.cax1a-12*. El objetivo de este estudio fue comprobar el efecto de estas mutaciones en la tolerancia a la sal, centrándose en la respuesta al proceso de fotosíntesis. Así, los tres mutantes *BraA.cax1a* y la línea parental (R-o-18) se cultivaron en condiciones de salinidad, y se midieron parámetros relacionados con la biomasa, el rendimiento de la fotosíntesis, la glucosa-6-fosfato deshidrogenasa (G6PDH, EC 1.1.1.49) y los carbohidratos solubles. *BraA.cax1a-4* proporcionó una mayor biomasa y un mejor rendimiento fotosintético manifestado por una mayor eficiencia en el uso del agua (WUE), y mayores valores de Fv/Fm, flujos de electrones y de Rubisco (EC 4.1.1.39). Además, *BraA.cax1a-4* presentó una mayor protección osmótica

mediante la acumulación de *myo*-inositol. Por otro lado, *BraA.cax1a-7* produjo algunos efectos negativos en el rendimiento de la fotosíntesis y menores acumulaciones de G6PDH y Rubisco. Por lo tanto, este estudio identifica a *BraA.cax1a-4* como una mutación útil para mejorar el rendimiento fotosintético en plantas cultivadas en condiciones salinas.

Manuscript submitted to Plant Science

Effect of CAX1a TILLING mutations on photosynthesis performance in salt-stressed *Brassica rapa* plants

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ABSTRACT

Salinity is an important environmental factor that reduces plant productivity in many world regions. It affects negatively photosynthesis causing a growth reduction. Likewise, calcium (Ca^{2+}) is crucial in plant stress response. Therefore, the modification of Ca^{2+} cation exchangers (CAX) transporters could be a potential strategy to increase plant tolerance to salinity. Using Targeting Induced Local Lesions in Genomes (TILLING), researchers generated three mutants of *Brassica rapa* CAX1a transporter: *BraA.cax1a-7, BraA.cax1a-4*, and *BraA.cax1a-12*. The aim of this study was to test the effect of those mutations on salt tolerance focusing on the response to the photosynthesis process. Thus, the three *BraA.cax1a* mutants and the parental line (R-o-18) were grown under salinity conditions, and parameters related to biomass, photosynthesis performance, glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), and soluble carbohydrates were measured. *BraA.cax1a-4* provided higher biomass and a better photosynthetic performance manifested by higher water use efficiency (WUE), Fv/Fm, electron fluxes, and Rubisco (EC 4.1.1.39) values. In addition, *BraA.cax1a-4* presented increased osmotic protection through *myo*-inositol accumulation. On the other hand, *BraA.cax1a-7* produced some negative effects

on photosynthesis performance and lower G6PDH and Rubisco accumulations. Therefore, this study points out *BraA.cax1a-4* as a useful mutation to improve photosynthetic performance in plants grown under saline conditions.

Keywords: *Brassica*, Carbohydrates, G6PDH, Photosynthesis, Rubisco, Salinity, TILLING

1. Introduction

One of the most pressing issues in the world, especially in the arid and semi-arid regions, influencing plant productivity is salinity. Higher concentrations of soluble salts either in soil or in irrigation water are the triggering factors of salinity. In recent years, this recurrent problem has caught the eye of many researchers, being predicted as the contributing factor in global warming (Acosta-Motos *et al.*, 2017). Several crops i.e. cash, cereals, or forage crops among others are prone to this problem. Brassicaceae family including cabbage, broccoli, and rapeseed are also included in this affected group. According to the previous studies, a lot of these species are cultivated in areas with higher salinity and the ones that are on the verge of being saline (Pavlović *et al.*, 2018). Among the possible causes of salinity, ion toxicity, mainly of Na^+, is the prominent one that interferes with vital processes within the plants (Negrão *et al.*, 2017).

One of the most important reasons for growth reduction in saline-stressed plants is that the photosynthesis process is altered either by the reduction in leaf

area or by the photosynthesis imbalance process at a cellular level. Thus, salinity stress causes direct salt toxicity and triggers leaf senescence producing first chlorosis and finally leaf abscission (Acosta-Motos *et al.*, 2017). In addition, the accumulation of salts in the growing medium reduces its osmotic potential, hindering water uptake by the roots (Kataria and Verma, 2018). Consequently, plants promote stomatal closure to reduce water loss. However, this closure limits CO_2 diffusion inside the leaf and the limitation in CO_2 supply inhibits the activity of the main enzyme for CO_2 assimilation, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) (Muhammad *et al.*, 2021). Besides, the regeneration of reducing power by the Calvin-Benson-Bassham cycle is hindered, which produces an imbalance between the photosystem (PS) II activity and the electron flow. This imbalance leads to an excessive photochemical energy accumulation that must be dissipated as fluorescence or may generate reactive oxygen species (ROS) that in turn produce photodamage (Rangani *et al.*, 2018). Furthermore, the excessive accumulation of Na^+ ions has direct negative effects on the components of the photosynthetic machinery, limiting its performance (Muhammad *et al.*, 2021).

Plants possess adaptation mechanisms to face salinity stress, including changes in ion homeostasis, water distribution, energy balance, and photosynthesis regulation. Plants regulate photosynthesis at different levels such as CO_2 assimilation, electron transport, or pigments distribution [7,8]. A good stomata regulation is crucial to maintain osmotic potential and avoid water loss by transpiration. Thus, plants with higher water use efficiency (WUE) present higher salt tolerance and a better stomatal aperture regulation to reduce water

loss whereas maintaining an adequate CO_2 flow into the leaf (Srinivas *et al.*, 2018). Another way to regulate osmotic potential is through the accumulation of osmolytes that decrease its value in the plant to allow water uptake. Examples of osmolytes are soluble carbohydrates such as glucose, sucrose, and inositol (Acosta-Motos *et al.*, 2017). Moreover, another mechanism that contributes to salinity tolerance is glucose oxidation in the pentose phosphate pathway (OPPP). In OPPP, glucose-6-phosphate is oxidized to pentose-P, generating NADPH and supplying carbon skeletons for the synthesis of nucleotides, lignin, coenzymes, and amino acids. This process is driven by glucose-6-phosphate dehydrogenase enzyme (G6PDH, EC 1.1.1.49), whose activity is enhanced under stress conditions to regulate energy balance and ensure NADPH supply for stress defense mechanisms and CO_2 assimilation (Esposito, 2016). Furthermore, the energy balance is also mediated by the distribution of components such as antenna pigments and other components of the electron transport chain of photosystems. The objective is to maximize energy uptake and transference whereas minimizing the energy losses in the form of heat or fluorescence (Kan *et al.*, 2017).

Besides, another important element involved in photosynthesis regulation is calcium (Ca^{2+}). Ca^{2+} is part of the oxygen-evolving complex and Ca^{2+} is necessary to activate electron transport chain proteins and Calvin-Benson-Bassham cycle enzymes (Hochmal *et al.*, 2015). Furthermore, Ca^{2+} fulfills crucial functions within plants such as maintaining membrane stability, osmotic balance and it is an important component in signaling processes. Indeed, Ca^{2+} ions act as signaling agents and their fluxes modulate plant responses to stress, regulating

metabolic processes (Pokotylo *et al.*, 2012). Likewise, Ca^{2+} is a Na^{+} antagonist and thereby Ca^{2+} has been considered crucial in plant adaptation to salinity (Park *et al.*, 2016). Salinity as several other stress processes induces higher cytosolic Ca^{2+} concentration to trigger stress responses. Cation exchangers (CAX) transporters are crucial in the modulation of Ca^{2+} gradients in cells as they are necessary to restore normal Ca^{2+} levels (Pokotylo *et al.*, 2012). It was observed different CAX activity in halophytes such as *Suaeda salsa* Pall., which could be key in salt tolerance (Han *et al.*, 2011). Hence, the generation of variations in the CAX transporter could be a potential strategy to increase plant tolerance to salinity (Pokotylo *et al.*, 2012).

TILLING (Targeting Induced Local Lesions in Genomes) technique has been used to generate modifications in CAX1 transporter. TILLING performs a screening of new variations in target genes that may be useful for the generation of plants with improved tolerance to stresses (Till *et al.*, 2003). Through TILLING, researchers generated three mutants of *Brassica rapa* ssp. trilocularis 'R-o-18' with modifications in CAX1a transporter; *BraA.CAX1a*: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12* (Lochlainn *et al.*, 2011). These mutations shift amino acids that could affect protein conformation and thus improve CAX1 function (Graham *et al.*, 2014). In this study, we test whether CAX modification could provide a better response to salinity given the close relationship between the activity of these transporters and the response to stresses such as salinity (Han *et al*., 2011; Pokotylo *et al*., 2012). Therefore, the hypothesis to test is that *BraA.CAX1a* modifications have an effect on the tolerance of these mutants

against salinity. In this work, we focus on the response to the photosynthesis process.

2. Material and methods

2.1. Plant material and growth conditions

The plant material used in this experiment was a parent line R-o-18 (without changes in BraA.CAX1a) of *B. rapa* ssp. trilocularis 'R-o-18' and three different mutants for BraA.CAX1a transporter: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12*. The amino acidic variations produced in BraA.CAX1a transporter were: *BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56). Mutant plants were developed and identified as described by Lochlainn et al. (2011) and Graham et al. (2014). The *B. rapa* seeds used in this study were obtained from the same plant population described in Graham *et al*. (2014) experiment. Seeds were germinated and placed in pots (13 cm x 13 cm x 12.5 cm) with vermiculite as substrate. Pots were placed in trays (55 cm x 40 cm x 8.5 cm). The trays with the pots were placed in a growth chamber with controlled environmental conditions: relative humidity 60-80%, temperature 22/18ºC (day/night), 14/10-h photoperiod, and 350 µmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR). Pots were watered with a nutritive solution composed of 4 mM KNO_3, 4 mM $Ca(NO_3)_2$ • 4 H_2O, 2 mM $MgSO_4$ • 7 H_2O, 6 mM KH_2PO_4, 1 mM NaH_2PO_4 • 2 H_2O, 2 µM $MnCl_2$ • 4 H_2O, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4$ • 5 H_2O, 0.1 µM Na_2MoO_4 • 2 H_2O, 5 µM Fe-chelate (Sequestrene; 138FeG100), and 10 µM H_3BO_3. The solution had a pH of 5.5–6.0 and was renewed every three days.

2.2. Treatments and experimental design

Treatments were started 30 days after germination and were applied for 21 days. Plants were subjected to two different treatments: Control (without NaCl added to the solution) and salinity (150 mM NaCl added to the solution). The two factors involved in the experiment were the salinity (S) and the mutant (M). The experimental design consisted of a randomized complete block with 8 treatments, 3 trays per treatment, and 8 plants per tray, thereby a total of 24 plants for each treatment and 3 replications each.

2.3. Plant sampling

Plant leaves were weighed to determine the fresh weight (FW) and then were washed with distilled water and dried on filter paper. A part of the leaves from each treatment was frozen at −40ºC for later biochemical assays and the other part was lyophilized to determine the dry weight (DW) and soluble carbohydrates. Leaf DW ratio was calculated as the ratio between leaf DW of control plants and leaf DW of plants treated with NaCl. Nine independent replicates from each treatment (n=9) were used for the analytical assays.

2.4. Pigment concentrations

Leaves (0.1 g) were grounded and 1 ml of Methanol was added for total chlorophyll (Chl) and carotenoid extraction. Then the mixture was centrifuged at 5000 × *g* for 5 min. Afterward, the absorbance of the supernatant was measured at three different wavelengths: 664, 648, and 470 nm. The Chl *a* and Chl *b* were

assessed using the Lichtenthaler equation (Hartmut and Lichtenthaler, 1987). Total Chl was calculated as the sum of Chl *a* and Chl *b*.

2.5. Fluorescence parameters

Plant leaves were kept in the dark during 30 min using special leaf clip holders. Chl *a* fluorescence kinetics was registered using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK); the fluorescence transients were induced by red light (650 nm) with 3000 µmol photons $m^{-2}s^{-1}$ light intensity. For each treatment, measurements were carried out in nine fully-expanded leaves at midstem position of different plants. Handy PEA software was used to obtain the values of all considered fluorescence parameters (Strasser *et al*. 2000).

2.6. G6PDH assay

For G6PDH extraction, 0.3 g of leaves were ground and suspended in 600 ml of a solution containing 50 mM Tris-HCl a pH 8.0, 10% glycerol, 15 mM $NADP^+$, 5 mM $MgCl_2$, 4 mM EDTA, and 1ml/30 gr Protease Inhibitor Cocktail (Sigma P9599). G6PDH activity was obtained as described previously by Castiglia *et al*. (2015) registering $NADP^+$ reduction at 340 nm using a Cary 60 spectrophotometer [Agilent Technologies, USA). The composition of the assay mixture was: the previously obtained extract, 50 mM Tris-HCl pH 8.0, 3 mM glucose-6P, 5 mM $MgCl_2$, 150 µM $NADP^+$. G6PDH activity was expressed as nmol NADPH min^{-1} mg^{-1} protein.

2.7. Western blotting

For Western blotting analysis, proteins were extracted following the same method as described by G6PDH assay. Then, proteins were separated by SDS-PAGE and transferred on a nitrocellulose membrane (Ge Healthcare) using the Transblot-turbo (Biorad, CA, Usa) as previously described (Cardi et al. 2016). Membranes were incubated with antibodies raised vs potato G6PDH isoforms (Wendt et al. 2000): cytosolic (Cyt-G6PDH), P1 (P1-G6PDH), and P2 (P2-G6PDH) and RuBISCO large subunit. After incubating the membrane with horseradish peroxidase (HRP)-linked secondary antibodies, cross-reacting polypeptides were identified by enhanced chemioluminescence (ECL) reaction (WesternBrightTM Quantum kit—Advansta, San Josè, CA, USA). The images were obtained by BioRad Chemidoc system (Bio-Rad, Hercules CA, USA). Control blot was performed using anti-tubulin antisera (Fig. S1).

2.8. Leaf gas exchange parameters

To register leaf gas exchange parameters an infra-red gas analyzer LICOR 6800 Portable Photosynthesis System (IRGA: LICOR Inc. Nebraska, USA) was used. Nine fully expanded leaves of nine different plants for each treatment were placed in a leaf cuvette set with optimal growth conditions. The instrument was warmed up for 30 min and calibrated before the measurements. Standard optimal cuvette conditions of 500 µmol mol^{-1} CO_2 concentration, 500 µmol m^2 s^{-1} PAR, 60% relative humidity, and 30°C leaf temperature. Net photosynthesis rate (A), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 (C_i) were registered. Data were analyzed using the “Photosyn Assistant” software.

Instantaneous water use efficiency (WUE) was calculated by dividing *A* by the corresponding *E*.

2.9. Soluble Carbohydrates analysis

Samples were lyophilized and soluble carbohydrates concentrations were determined in the Metabolomics Service of CEBAS-CSIC (Murcia, Spain) using ^{1}H-NMR technique and according to the methodology described in Massaretto *et al*. (2018).

2.10. Statistical analysis

The mean and standard error of each treatment was obtained from the 9 individual data of each parameter considered. A one-way analysis of variance (ANOVA) with 95% confidence was performed using the Statgraphics Centurion 16.1.03 software. A two-tailed ANOVA was applied to evaluate whether the saline treatment (S), the mutations (M), or the interaction (S * M) significantly influenced the results. Means were compared by Fisher's least significant differences (LSD). The significance levels for both analyses were expressed as * $P<0.05$, ** $P<0.01$, *** $P<0.001$, or NS (not significant).

3. Results

3.1. Plant biomass

The different *B. rapa* lines did not show significant differences in leaf DW under control conditions (Fig. 1A). The application of the NaCl treatments

negatively affected plants, which was evidenced by smaller plants, with the presence of chlorosis in the leaves and a remarkable decrease in leaf DW in comparison to control plants. Nevertheless, this reduction was lower in *BraA.cax1a-4* plants that presented higher leaf DW than the other lines (Fig. 1A). Furthermore, considering the ratio between leaf DW of control plants and leaf DW of plants treated with NaCl, we observed lower values in *BraA.cax1a* mutants than in R-o-18 plants (Fig. 1B).

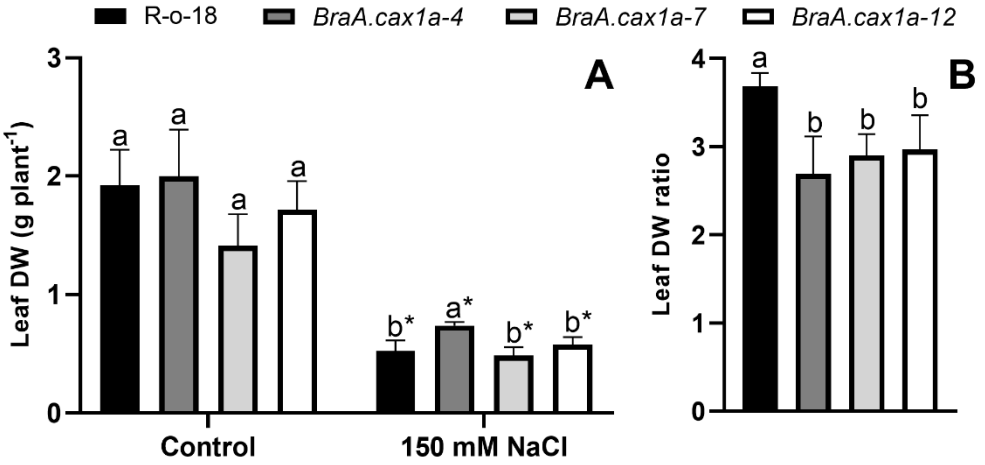


Figure 1. Effect of *BraA.cax1a* mutations and NaCl treatment on leaf DW (A) and leaf DW ratio (B). Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$). Asterisk (*) indicates significative differences between control and 150 mM treatments.

3.2. Photosynthetic pigments concentration

Under control conditions, *BraA.cax1a* mutants showed lower total Chls concentration in comparison to R-o-18 plants. Furthermore, under saline conditions, the level of this parameter was lower in all lines compared to that of plants not supplied with NaCl. Comparing among lines, *BraA.cax1a-7* plants showed lower Chls levels than R-o-18, whereas the other mutants did not show significant differences in comparison to R-o-18 plants. However, *BraA.cax1a*

plants presented higher Chl *a*/*b* ratios compared to R-o-18 plants. Like Chls, carotenoids were less concentrated in *BraA.cax1a* plants being *BraA.cax1a-7* the mutant with the lowest carotenoids concentration (Table 1).

Table 1. SPAD value, total Chls concentration, Chl a/b ratio and carotenoids concentration in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		Total Chls mg g^{-1} FW	Chl *a*/*b*	Carotenoids µg g^{-1} FW
Control	R-o-18	0.73a	2.30b	52.05a
	BraA.cax1a-4	0.67b	2.37a	49.10ab
	BraA.cax1a-7	0.61b	2.24c	49.34ab
	BraA.cax1a-12	0.63b	2.31b	45.88b
	p-value	**	***	NS
	$LSD_{0.05}$	0.06	0.03	5.36
150 mM NaCl	R-o-18	0.63a	2.17b	52.19a
	BraA.cax1a-4	0.59a	2.29a	43.75b
	BraA.cax1a-7	0.53b	2.29a	39.54c
	BraA.cax1a-12	0.60a	2.33a	43.88b
	p-value	***	***	***
	$LSD_{0.05}$	0.05	0.05	3.82
Analysis of variance				
Salinity (S)		***	***	***
Mutation (M)		***	***	***
S x M		NS	***	*
$LSD_{0.05}$		0.03	0.03	3.19

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.3. Chl a fluorescence parameters

Comparing the different lines grown under control conditions, *BraA.cax1a-7* mutants registered higher Fo, Fm, and K step, and lower Fv/Fm and RC/ABS

values than R-o-18 plants, whereas the rest of the lines showed similar values. Regarding salinity conditions, R-o-18 and *BraA.cax1a-12* plants showed higher Fo values, whereas all plants subjected to salinity presented lower Fm values in comparison to control conditions. Although *BraA.cax1a-7* plants registered the highest values for these parameters and *BraA.cax1a-4* plants presented the lowest Fo in comparison to the other lines. Salinity stress reduced the Fv/Fm values of all plants compared to those of plants not supplied with NaCl, but *BraA.cax1a-4* plants reached higher Fv/Fm values than the other lines. About Ψ_{Eo} and Φ_{Eo} parameters, it was not observed significant effects of salinity nor mutations. Furthermore, salinity reduced PI_{ABS} value in all plants except in *BraA.cax1a-4* plants in comparison to control conditions but no different PI_{ABS} values were observed comparing among the four lines. Salinity reduced RC/ABS values in all *B. rapa* genotypes, whereas *BraA.cax1a-7* plants showed lower RC/ABS in comparison to R-o-18 plants. In contrast, salinity increased K step values in all the lines compared to plants that did not received NaCl, and *BraA.cax1a-7* reached the higher K step value compared to R-o-18 plants. In addition, NaCl application increased t Fm values of R-o-18 and *BraA.cax1a-7* plants in comparison to control conditions and all *BraA.cax1a* plants showed higher t Fm values compared to R-o-18 plants (Table 2).

Table 2. Photosynthetic fluorescence parameters in R-o-18 and *BraA.cax1a* mutants grown under control ar

		Fo	Fm	Fv/Fm	Ψ_{Eo}	Φ_{Eo}	PI_{ABS}	RC/AB
Control	R-o-18	349b	2203b	0.841a	0.66a	0.56a	9.82a	1.00a
	BraA.cax1a-4	366b	2232b	0.836a	0.66a	0.55a	8.61a	0.94ab
	BraA.cax1a-7	465a	2579a	0.821b	0.65a	0.51a	8.99a	0.87b
	BraA.cax1a-12	351b	2207b	0.841a	0.62a	0.52a	9.61a	0.96a
	p-value	**	*	***	NS	NS	NS	**
	LSD0.05	67	282	0.012	0.07	0.07	1.37	0.08
150 mM NaCl	R-o-18	395bc	1937ab	0.796b	0.68a	0.56a	8.76a	0.90a
	BraA.cax1a-4	359c	1828b	0.804a	0.66a	0.55a	8.56a	0.88a
	BraA.cax1a-7	417a	2063a	0.798ab	0.68a	0.56a	7.84a	0.69b
	BraA.cax1a-12	393ab	1966ab	0.801ab	0.65a	0.53a	8.68a	0.81a
	p-value	**	*	*	NS	NS	NS	***
	LSD0.05	30	142	0.006	0.06	0.05	1.56	0.09
Analysis of variance								
Salinity (S)		***	***	***	NS	NS	*	***
Mutation (M)		***	**	***	NS	NS	NS	***
S x M		NS	NS	***	NS	NS	NS	NS
LSD0.05		35	153	0.007	0.05	0.04	0.98	0.06

Values are means ± standard error (n=9) and differences between means were compared by Fisher's le
Values with different letters indicate significant differences. The levels of significance were represented by
(*), p<0.01 (**) and p<0.001 (***).

Regarding electron fluxes, *BraA.cax1a* plants presented higher ABS/RC, TRo/RC, and ETo/RC values, and *BraA.cax1a-7* plants registered higher DIo/RC value in comparison to the R-o-18 line when they were grown without NaCl. Under saline conditions, all lines showed a similar decrease caused by salinity in ABS/RC, TRo/RC, and ETo/RC parameters in comparison to control conditions, although *BraA.cax1a* mutants register higher values than R-o-18 plants (Fig. 2A-C). Besides, salinity increase DIo/RC value in *BraA.cax1a-4* and especially in the *BraA.cax1a-7* line compared to plants without NaCl. Indeed, under saline conditions, *BraA.cax1a-7* registered the highest DIo/RC value in comparison to the other lines (Fig. 2D).

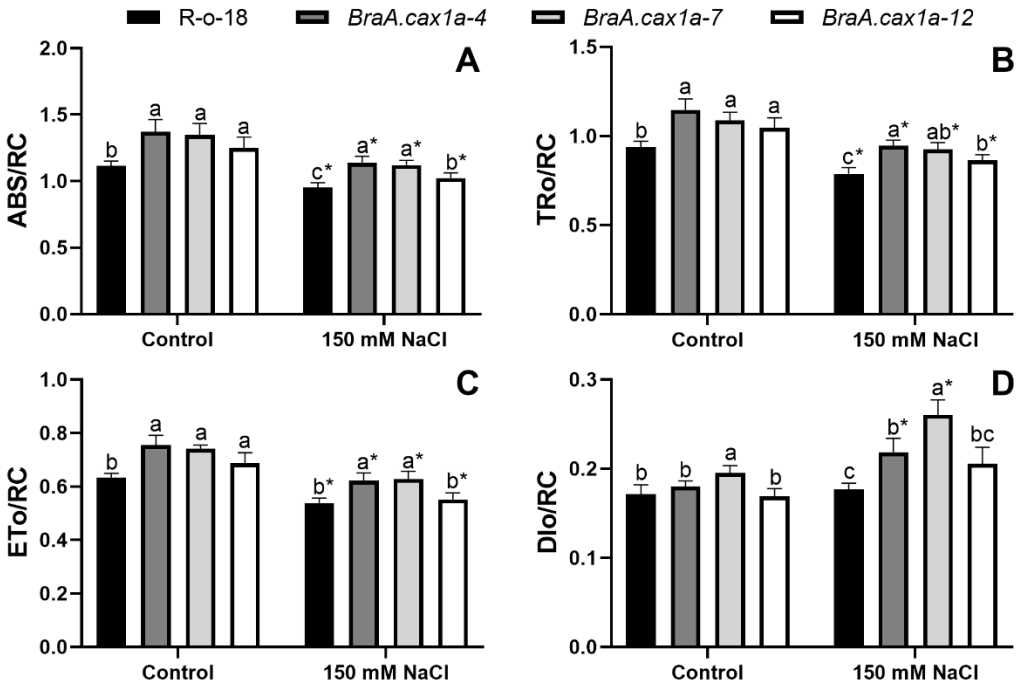


Figure 2. Effect of *BraA.cax1a* mutations and NaCl treatment on photosynthetic electron fluxes. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$). Asterisk (*) indicates significative differences between control and 150 mM treatments.

3.4. G6PDH and Rubisco

Salinity did not affect G6PDH activity in *B. rapa* plants except in *BraA.cax1a-7* plants that showed an increment in comparison to plants not supplied with NaCl. However, this mutant showed the lowest G6PDH activity, whereas *BraA.cax1a-4* reached the highest G6PDH activity compared to the other lines grown under both saline and control conditions (Fig. 3). Furthermore, western blot analysis showed lower Cyt-G6PDH and Rubisco accumulations in the *BraA.cax1a-7* line under control conditions. R-o-18 and *BraA.cax1a-4* plants showed a reduction in Cyt-G6PDH and Rubisco presence caused by NaCl, whereas in the *BraA.cax1a-7* and *BraA.cax1a-12* lines the accumulation of these two elements increased compared to plants not grown under salinity. Comparing among lines, *BraA.cax1a-4* and *BraA.cax1a-12* plants showed higher Cyt-G6PDH and Rubisco accumulations, whereas *BraA.cax1a-7* registered lower accumulation of these enzymes compared to R-o-18 plants under saline conditions (Fig. 4A and 4D). With reference to P1-G6PDH isoform, all *BraA.cax1a* plants showed higher accumulation than R-o-18 plants under no salinity conditions. Nevertheless, P1-G6PDH accumulation was reduced by salinity in *BraA.cax1a* plants compared to control conditions. Under saline conditions, the presence of this isoform was lower in *BraA.cax1a-4* but higher in *BraA.cax1a-7* and *BraA.cax1a-12* plants in comparison to R-o-18 plants (Fig. 4B). Besides, *BraA.cax1a-4* showed lower accumulation of P2-G6PDH isoform, whereas *BraA.cax1a-7* and *BraA.cax1a-12* plants did not show significant changes compared to control conditions. Comparing among genotypes, *BraA.cax1a-4* showed higher P2-G6PDH,

whereas the presence of this isoform was lower in *BraA.cax1a-7* compared to R-o-18 plants.

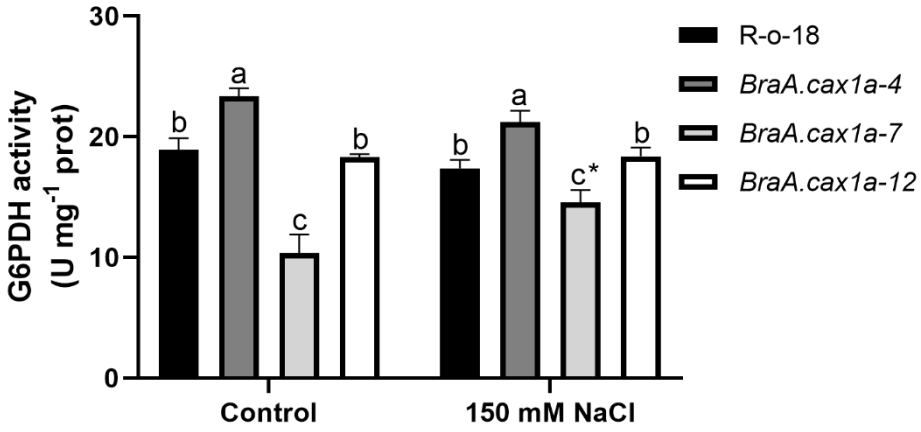


Figure 3. Effect of *BraA.cax1a* mutations and NaCl treatment on G6PDH activity. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test ($P < 0.05$). Asterisk (*) indicates significative differences between control and 150 mM treatments.

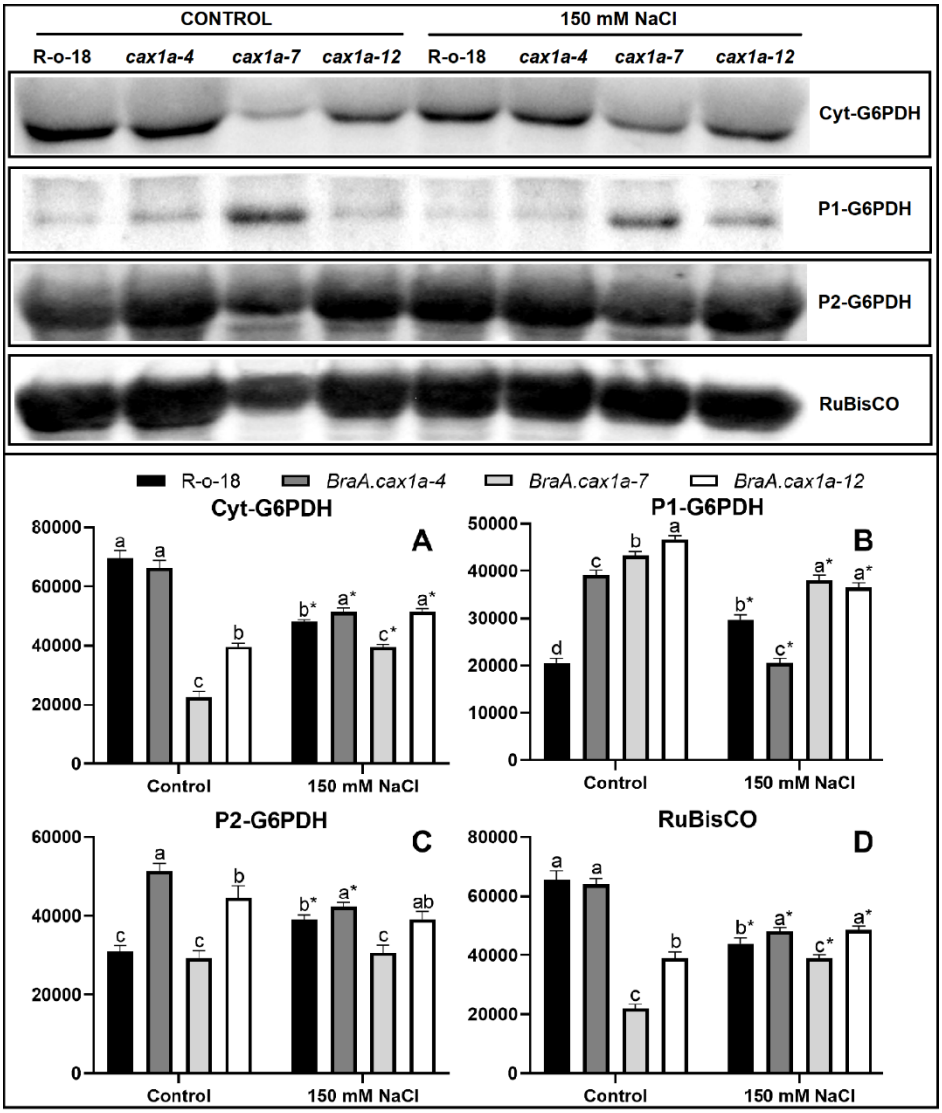


Figure 4. Effect of *BraA.cax1a* mutations and NaCl treatment on the accumulation of G6PDH and RuBisCO. The upper panel shows western blotting. Lower panel shows densitometric analysis using Image J software.

3.5. Gas exchange parameters

In control plants, no differences among lines were observed regarding *A*, whereas the *BraA.cax1a-12* line registered higher *E*, g_s, and *C*i and lower WUE compared to R-o-18 plants. Moreover, NaCl application reduced *A*, *E*, g_s in *B. rapa* plants in comparison to control conditions. Comparing among lines, *BraA.cax1a-4* and *BraA.cax1a-7* showed higher *A*, *E*, and g_s than R-o-18 plants.

Regarding *C*i, no significant effects of salinity were observed in *BraA.cax1a* plants compared to control conditions and no differences between lines grown under salinity were observed. In addition, water use efficiency (WUE) was lower in all the lines subjected to salinity, except *BraA.cax1a-12*, in comparison to plants not supplied with NaCl. Furthermore, *BraA.cax1a-4* plants registered higher WUE compared to the other lines (Table 3).

Table 3. Gas exchange parameters in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		A (µmol m^{-2} s^{-1})	*E* (mmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (µmol mol^{-1})	WUE (µmol $mmol^{-1}$)
Control	R-o-18	8.99a	2.89b	0.18b	375b	3.15a
	BraA.cax1a-4	9.20a	3.15b	0.24b	402ab	2.96ab
	BraA.cax1a-7	8.88a	4.33ab	0.35ab	428a	2.42b
	BraA.cax1a-12	8.80a	5.69a	0.47a	419a	1.31c
	p-value	NS	*	*	*	***
	$LSD_{0.05}$	1.51	2.23	0.21	39.93	0.55
150 mM NaCl	R-o-18	1.35b	0.60c	0.04c	431a	1.66b
	BraA.cax1a-4	2.16a	0.77ab	0.05ab	418a	2.11a
	BraA.cax1a-7	2.02a	1.06a	0.07a	426a	1.62b
	BraA.cax1a-12	1.77ab	0.88bc	0.04bc	406a	2.02ab
	p-value	*	*	**	NS	*
	$LSD_{0.05}$	0.67	0.27	0.01	38.26	0.42
Analysis of variance						
Salinity (S)		***	***	***	NS	***
Mutation (M)		*	*	*	NS	***
S x M		NS	NS	*	NS	***
$LSD_{0.05}$		0.78	1.06	0.1	26.19	0.34

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

3.6. Soluble carbohydrates concentration

BraA.cax1a-7 plants presented lower glucose, fructose, and sucrose concentrations, whereas *BraA.cax1a-4* and *BraA.cax1a*-12 registered lower *myo*-inositol concentration compared to R-o-18 plants when NaCl was not applied. Regardless of the genotype, salinity caused an increase in all the analyzed carbohydrates except sucrose in comparison to control conditions. Comparing among lines, *BraA.cax1a* plants showed lower glucose and *myo*-inositol concentrations in comparison to R-o-18 plants, highlighting *BraA.cax1a-7* plants that presented the lowest values. Besides, *BraA.cax1a-4* and specially *BraA.cax1a-7* plants registered lower fructose concentration compared to R-o-18 plants. However, *BraA.cax1a-7* was the unique line presenting measurable levels of sucrose under saline conditions (Table 4).

Table 4. Carbohydrates concentration in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

		Glucose mg g^{-1} DW	Fructose mg g^{-1} DW	Sucrose (mg g^{-1} DW)	*Myo*-inositol (mg g^{-1} DW)
Control	R-o-18	1.74b	5.36b	0.12b	2.62a
	BraA.cax1a-4	1.14c	7.38a	0.13b	1.92c
	BraA.cax1a-7	1.22c	4.56c	0.10c	2.49a
	BraA.cax1a-12	2.03a	4.36c	0.31a	2.16b
	p-value	***	***	***	***
	$LSD_{0.05}$	0.15	0.52	0.02	0.22
150 mM NaCl	R-o-18	7.17a	16.74a	nd	6.16a
	BraA.cax1a-4	2.78c	12.46b	nd	4.97b
	BraA.cax1a-7	1.71d	8.84c	0.03	4.45c
	BraA.cax1a-12	4.99b	17.85a	nd	5.27b
	p-value	***	***		***
	$LSD_{0.05}$	0.44	1.36		0.41
Analysis of variance					
Salinity (S)		***	***	***	***
Mutation (M)		***	***	***	***
S x M		***	***	***	***
$LSD_{0.05}$		0.21	0.67	0.01	0.21

Values are means ± standard error (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values below the detection limit are indicated as nd. Values with different letters indicate significant differences. The level of significance were represented by p<0.001 (***).

4. Discussion

The most immediate effect of salinity on plants and specifically in *Brassica* crops is that they do not reach their potential biomass due to they are considered species with moderate sensitivity to saline stress (Pavlović *et al.*, 2018). Thus, in this present experiment, all plants showed a significant reduction in plant growth compared to control conditions although this diminution was greater in the R-o-18 plants than in *BraA.cax1a* plants as shown by the leaf DW ratio results. This

result suggests an influence of CAX1 transporter on plant growth in *B. rapa* plants grown under saline conditions. However, comparing between genotypes, only *BraA.cax1a-4* plants registered higher biomass than R-o-18 plants under saline stress. Thereby, *BraA.cax1a-4* mutation could provide greater tolerance to high NaCl concentration in the growth medium. The higher Ca^{2+} and K^+ contents and the lower Na^+/K^+ ratio observed in a previous study in *BraA.cax1a-4* (Navarro-León *et al.*, 2020) could contribute to the higher NaCl tolerance and also could have a positive effect on photosynthetic performance.

Several experiments showed that salinity also causes a reduction in the concentration of photosynthetic pigments in plants (Acosta-Motos *et al.*, 2017; Cantabella *et al.*, 2017; Parida and Das, 2005) . Actually, the chlorophyll content decreases more in salt-sensitive species, whereas it can even increase in salt-tolerant species (Acosta-Motos *et al*., 2017; Negrão *et al*. 2017). In the present experiment, all *B. rapa* lines showed a decrease in total Chls concentration caused by salinity. Although this decrease in Chls concentration was lower in *BraA.cax1a-12* plants, this mutation did not provide higher salinity tolerance. Furthermore, other studies observed that Ca application enhances Chl *a* concentration in plants grown under saline conditions (García-Martí *et al.*, 2019; Mukhtar *et al.*, 2016). Nevertheless, *BraA.cax1a*-7 had negative effect on Chl concentration, although it was not determinant for lower biomass because this mutant showed a similar biomass loss than the other lines.

Stresses such as salinity usually increase the proportion of Chl *b* over Chl *a* leading to a lower Chl *a*/*b* ratio (Cantabella *et al.*, 2017). In chloroplast, Chl *a* is principally presented in reactive centers (RCs) and core antenna proteins of PS II, whereas Chl *b* is located mainly in light-harvesting complexes. A higher Chl *a*/*b* ratio usually is indicative of the conversion of Chl *b* into Chl *a* to maintain active RCs under stress (Nyongesah *et al.*, 2015; Yang *et al.*, 2009). This conversion could be the cause of the higher Chl *a*/*b* ratio observed in *BraA.cax1a* mutants. Alternatively, carotenoids act as accessory pigments, light-harvesting compounds, and as antioxidants (Rangani *et al.*, 2018). Regarding carotenoids, other experiments measured either no effects or a decrease in their concentrations under saline conditions (Parida and Das, 2005). However, in this study salinity only produced a clear reduction in *BraA.cax1a-7* plants, although all *BraA.cax1a* reduced its concentration. Hence, changes in Ca^{2+} fluxes presumably induced by *BraA.cax1a* mutations could influence the synthesis of leaf photosynthetic pigments in plants subjected to salinity in a non-determinant way on growth. This influence of Ca^{2+} fluxes probably would be mediated by Ca-dependent kinases such as NADK2 that was proven to be crucial in chlorophyll synthesis (Chai *et al.*, 2005).

Photosynthetic pigments, in addition to transferring useful energy for photosynthesis, emit back as fluorescence a part of the energy absorbed. This fluorescence emission is higher under stress conditions. Thus, Chl *a* fluorescence is a good method to evaluate PS II performance and the alterations in photosynthesis caused by stress (Shu *et al.*, 2012). Derived from Chl *a* fluorescence analysis, Fv/Fm and PI_{ABS} parameters are reliable indicators of PS

II performance that usually are reduced in plants grown under saline stress (Chrysargyris *et al.*, 2019; Kan *et al.*, 2017). Indeed, a decrease in these parameters was observed in another experiment in *B. rapa* plants grown with 200 mM NaCl, although this reduction was not produced when 100 mM NaCl was applied (Pavlović *et al.*, 2018). In the present experiment, using 150 mM NaCl, we observed a reduction in Fv/Fm and PI_{ABS} parameters and also negative effects were produced on other fluorescence parameters such as RC/ABS, K step, and electron fluxes caused by salinity, which indicates an impairment in energy transference within photosystems (Strasser *et al.*, 2004). Comparing lines, *BraA.cax1a-4* showed better photosynthetic performance because registered higher Fv/Fm and electron fluxes values. Although *BraA.cax1a-7* also presented higher fluxes, it showed the highest DIo/RC value, which suggests a higher wasting of photosynthetic energy. Furthermore, this mutant showed lower RC/ABS and higher K step values suggesting a worse photosynthetic performance. Zhao et al. (Zhao *et al.*, 2019) study compared maize genotypes with different salt tolerance and observed that differences in CAX1 transporter activity provided different PS performance, which could explain the differences observed in *BraA.cax1a* mutants. Changes in CAX1 could affect Ca^{2+} fluxes and profiles in cells, which could explain the effect on photosynthetic performance produced in *BraA.cax1a* mutants because of the great importance of Ca^{2+} in photosynthesis regulation directly or through Ca-dependent regulator proteins (Hochmal *et al.*, 2015).

Another process related to energy supply is OPPP in which glucose-6-phosphate (G6P) is oxidized to pentose-P, generating NADPH. The key enzyme

in this process is G6PDH (Esposito, 2016). In salt-stress conditions, G6PDH activity is enhanced to supply the demand for reductants for antioxidant systems and other stress responses (Landi *et al.*, 2016). In contrast, this induction of G6PDH activity was not observed as a result of salinity stress in our experiment. However, *BraA.cax1a-4* plants showed the highest G6PDH activity under salinity conditions, which could contribute to a higher NADPH generation to face oxidative stress. On the other hand, the lower G6PDH activity in *BraA.cax1a-7* might cause a NADPH shortage, thereby explaining its worse photosynthetic performance. This effect of *BraA.cax1a* mutations could be due to Ca^{2+} is involved in G6PDH activation (Semenikhina *et al.*, 1999) and thereby the probably different Ca^{2+} profiles generated in the mutants could have influence on G6PDH activity.

G6PDH enzyme has three main isoforms in plants. The Cyt-G6PDH isoform represents about 60-85% of all G6PDH activity. The other isoforms are P1-G6PDH (chloroplastic isoform) and P2-G6PDHs (plastidic isoform). Considering these isoforms, Cyt-G6PDH accumulation was well correlated with G6PDH activity agreeing with the fact that cytosolic isoform is responsible for the main G6PDH activity in cells. Alternatively, P1-G6PDH functions in the dark to generate NADPH to maintain basal C metabolism (Esposito, 2016). In *B. rapa* plants grown under salinity, P1-G6PDH accumulation showed a particular pattern because *BraA.cax1a-7* showed the highest P1-G6PDH probably to offset its lower photosynthesis performance and to maintain NADPH generation in chloroplasts. Interestingly, under control conditions in *BraA.cax1a-7* the increased occurrence of P1-G6PDH corresponds to lower levels of P2-G6PDH,

suggesting a compensation effect; on the other hand, salinity increased specifically the plastidic P2-G6PDH, supporting the hypothesis that this isoform is able to sustain the increased requirement of reductants under stress (Cardi *et al.*, 2011; Landi *et al.*, 2016).

An adequate NADPH supply is curcial for optimal plant funcitioning and to avoid imbalances between photochemical phase of photosynthesis and Calvin-Benson-Bassham cycle. The main enzyme in this cycle is Rubisco, which is a key enzyme in photosynthesis that fixes CO_2 in plants and its activity is regulated under stress occurrence (Muhammad *et al.*, 2021). Thus, concentrations above 100 mM NaCl reduce Rubisco activity and accumulation. Furthermore, its activity is closely related to the availability of CO_2 in photosynthetic tissues (Galmés *et al.*, 2013; Sarabi *et al.*, 2019). NaCl could difficult CO_2 diffusion in mesophyll regardless of stomata closure and thereby reduce Rubisco activity (Parida and Das, 2005). However, in the present study, Rubisco accumulation was not correlated with *C*i plants subjected to salinity. Therefore, the difference in Rubisco accumulation observed between the different lines might be related to the effect of Ca^{2+} regulation. Thus, Ca^{2+} signaling is crucial for the enzyme activities of Calvin-Benson-Bassham such as Rubisco (Hochmal *et al.*, 2015). Similar to other photosynthetic parameters, *BraA.cax1a-4* showed higher Rubisco accumulation and this enzyme was less accumulated in *BraA.cax1a-7* plants. Higher Rubisco availability could be key to sustain the higher biomass observed in *BraA.cax1a-4* plants grown under saline conditions.

Rubisco enzyme needs a good CO_2 supply for optimal activity. To estimate CO_2 supply and also H_2O fluxes, we analyzed gas exchange parameters, which are useful indicators to evaluate the photosynthesis and also the transpiration processes in photosynthetic organisms. Likewise, changes in leaf gas exchanger parameters are good indicators of stress in plants. For instance, salinity usually reduces the values of these parameters in plants (Muhammad *et al.*, 2021). However, several authors observed that Ca application restored gas exchange values, which was correlated with a growth improvement (García-Martí *et al.*, 2019; Mukhtar *et al.*, 2016; Sahin *et al.*, 2018). We observed that salinity reduced the values of gas exchange parameters of all the analyzed plants but to a lesser extent in *BraA.cax1a-4* and *BraA.cax1a-7* plants. Indeed, these plants presented higher *A* than R-o-18, although this increment only was translated in higher biomass in *BraA.cax1a-4* plants. Probably, other factors such as the higher water loss by transpiration, reflected in higher *E* and g_s values, and the lower WUE influenced negatively to *BraA.cax1a-7* plants. Despite *BraA.cax1a-4* plants also showed higher E and g_s values, they presented a greater WUE value, which suggests a better stomata regulation to avoid excessive water loss but maintaining CO_2 capture. Ca^{2+} is involved in stomatal response (Cantabella *et al.*, 2017), which may explain the effect of salinity and *BraA.cax1a* mutations on g_s and transpiration.

Soluble carbohydrates, besides being the direct result of the photosynthetic process, generally are accumulated in plants as a consequence of salt stress. Thus, compounds such as glucose, fructose, sucrose, and *myo*-inositol act as osmoprotectors, carbon storage, and also radical scavenging (Muhammad *et al.*,

2021; Sahin *et al.*, 2018). The analysis of soluble carbohydrates in *B. rapa* plants showed an increase in their concentrations caused by salinity. Furthermore, Ca^{2+} is related to soluble carbohydrates accumulation and the enzyme activities for their synthesis and catabolism (Duan *et al.*, 2020; Hochmal *et al.*, 2015). This fact could explain the lower soluble carbohydrates concentration observed in *BraA.cax1a* plants. Among them, *BraA.cax1a-7* plants showed the lowest monosaccharides concentrations, which might be caused by a lower carbohydrate synthesis derived from its worse photosynthetic performance. However, the higher sucrose concentration in *BraA.cax1a-7* plants might be related to higher carbohydrate remobilization, being that sucrose fulfills this function in stressed plants (Perri *et al.*, 2019). Regarding *myo*-inositol, it plays an important role in salt stress response acting as an osmolyte and is accumulated in halophytes (Hazra and Nandy, 2016). In addition, the increment in *myo*-inositol levels in salt-stressed plants has proved to be an effective strategy to provide higher salt tolerance (Al Hassan *et al.*, 2016; Hu *et al.*, 2018). Thus, in the present study, the 159% increment of myo-inositol concentration in *BraA.cax1a-4* plants could be key to the higher tolerance observed in this line.

5. Conclusion

In conclusion, salinity application caused stress to *B. rapa* plants manifested by a decrease in biomass and a negative effect on photosynthetic performance. However, *BraA.cax1a* mutations influenced *B. rapa* response to salinity. Thus, changes in CAX1 transporter could affect cellular Ca^{2+} fluxes that mediate the regulation of the different parameters analyzed in this study. Indeed, *BraA.cax1a-4* mutation provided higher biomass and better photosynthetic performance.

Thus, these plants showed better stomata regulation, electron fluxes, and higher Rubisco accumulation. In addition, *BraA.cax1a-4* could present higher NADPH production through G6PDH activity and greater osmotic protection through *myo*-inositol accumulation. On the other hand, *BraA.cax1a-7* mutation produces negative effects on photosynthesis performance, such as a higher energy loss through fluorescence and a worse electron transport efficiency and lower G6PDH activity, and Rubisco and monosaccharides accumulations. Therefore, this study points out *BraA.cax1a-4* as a useful mutation to improve photosynthetic performance in plants grown under saline conditions, although further studies are necessary to elucidate the specific mechanisms of this tolerance.

Acknowledgments

We thank Dr. Martin R. Broadley and Dr. Neil Graham from Nottingham University for providing us the seeds employed in this experiment, and Dr. Enrique Pérez Sánchez-Cañete and Sergio Aranda-Barranco for the help with gas exchange measurements.

Funding

This work was supported by the PAI program (Plan Andaluz de Investigación, Grupo de Investigación AGR282) and by a Grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL [FPU14/01858].

References

Acosta-Motos, J., Ortuño, M., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M., Hernandez, J., 2017. Plant responses to salt stress: Adaptive mechanisms. Agronomy 7, 18. https://doi.org/10.3390/agronomy7010018

Al Hassan, M., Morosan, M., López-Gresa, M., Prohens, J., Vicente, O., Boscaiu, M., 2016. Salinity-induced variation in biochemical markers provides insight into the mechanisms of salt tolerance in common (*Phaseolus vulgaris*) and runner (*P. coccineus*) beans. Int. J. Mol. Sci. 17, 1582. https://doi.org/10.3390/ijms17091582

Ashraf, M., Harris, P.J.C., 2013. Photosynthesis under stressful environments: An overview. Photosynthetica 51, 163–190. https://doi.org/10.1007/s11099-013-0021-6

Cantabella, D., Piqueras, A., Acosta-Motos, J.R., Bernal-Vicente, A., Hernández, J.A., Díaz-Vivancos, P., 2017. Salt-tolerance mechanisms induced in Stevia rebaudiana Bertoni: Effects on mineral nutrition, antioxidative metabolism and steviol glycoside content. Plant Physiol. Biochem. 115, 484–496. https://doi.org/10.1016/j.plaphy.2017.04.023

Cardi, M., Chibani, K., Cafasso, D., Rouhier, N., Jacquot, J.-P., Esposito, S., 2011. Abscisic acid effects on activity and expression of barley (*Hordeum vulgare*) plastidial glucose-6-phosphate dehydrogenase. J. Exp. Bot. 62, 4013–4023. https://doi.org/10.1093/jxb/err100

Cardi, M., Zaffagnini, M., De Lillo, A., Castiglia, D., Chibani, K., Gualberto, J.M., Rouhier, N., Jacquot, J.-P., Esposito, S., 2016. Plastidic P2 glucose-6P dehydrogenase from poplar is modulated by thioredoxin m -type: Distinct roles of cysteine residues in redox regulation and NADPH inhibition. Plant Sci. 252, 257–266. https://doi.org/10.1016/j.plantsci.2016.08.003

Castiglia, D., Cardi, M., Landi, S., Cafasso, D., Esposito, S., 2015. Expression and characterization of a cytosolic glucose 6 phosphate dehydrogenase isoform from barley (*Hordeum vulgare*) roots. Protein Expr. Purif. 112, 8–14. https://doi.org/10.1016/j.pep.2015.03.016

Chrysargyris, A., Solomou, M., Petropoulos, S.A., Tzortzakis, N., 2019. Physiological and biochemical attributes of *Mentha spicata* when subjected to saline conditions and cation foliar application. J. Plant Physiol. 232, 27–38. https://doi.org/10.1016/j.jplph.2018.10.024

Duan, S., Wu, Y., Zhang, Chengjun, Wang, L., Song, S., Ma, C., Zhang, Caixi, Xu, W., Bondada, B., Wang, S., 2020. Differential regulation of enzyme activities and physio-anatomical aspects of calcium nutrition in grapevine. Sci. Hortic. (Amsterdam). 272, 109423. https://doi.org/10.1016/j.scienta.2020.109423

Esposito, S., 2016. Nitrogen Assimilation, Abiotic Stress and Glucose 6-Phosphate Dehydrogenase: The Full Circle of Reductants. Plants 5, 24. https://doi.org/10.3390/plants5020024

Galmés, J., Aranjuelo, I., Medrano, H., Flexas, J., 2013. Variation in Rubisco content and activity under variable climatic factors. Photosynth. Res. 117, 73–90. https://doi.org/10.1007/s11120-013-9861-y

García-Martí, M., Piñero, M.C., García-Sanchez, F., Mestre, T.C., López-Delacalle, M., Martínez, V., Rivero, R.M., 2019. Amelioration of the oxidative stress generated by simple or combined abiotic stress through the K^+ and Ca^{2+} supplementation in tomato plants. Antioxidants 8, 81. https://doi.org/10.3390/antiox8040081

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of Brassica under altered Ca supply identifies *Arabidopsis* Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Han, N., Shao, Q., Bao, H., Wang, B., 2011. Cloning and characterization of a Ca^{2+}/H^+ antiporter from halophyte *Suaeda salsa* L. Plant Mol. Biol. Report. 29, 449–457. https://doi.org/10.1007/s11105-010-0244-7

Hartmut, A., Lichtenthaler, K., 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382. https://doi.org/10.1016/0076-6879(87)48036-1

Hazra, A., Nandy, P., 2016. Myo-inositol 1-phosphate synthase – the chosen path of evolution. BioTechnologia 2, 95–108. https://doi.org/10.5114/bta.2016.60780

Hochmal, A.K., Schulze, S., Trompelt, K., Hippler, M., 2015. Calcium-dependent regulation of photosynthesis. Biochim. Biophys. Acta - Bioenerg. 1847, 993–1003. https://doi.org/10.1016/j.bbabio.2015.02.010

Hu, L., Zhou, K., Li, Y., Chen, X., Liu, B., Li, C., Gong, X., Ma, F., 2018. Exogenous myo-inositol alleviates salinity-induced stress in Malus hupehensis Rehd. Plant Physiol. Biochem. 133, 116–126. https://doi.org/10.1016/j.plaphy.2018.10.037

Kan, X., Ren, J., Chen, T., Cui, M., Li, C., Zhou, R., Zhang, Y., Liu, H., Deng, D., Yin, Z., 2017. Effects of salinity on photosynthesis in maize probed by prompt fluorescence, delayed fluorescence and P700 signals. Environ. Exp. Bot. 140, 56–64. https://doi.org/10.1016/j.envexpbot.2017.05.019

Kataria, S., Verma, S.K., 2018. Salinity Stress Responses and Adaptive Mechanisms in Major Glycophytic Crops: The Story So Far, in: Salinity Responses and Tolerance in Plants, Volume 1. Springer International Publishing, Cham, pp. 1–39. https://doi.org/10.1007/978-3-319-75671-4_1

Landi, S., Nurcato, R., De Lillo, A., Lentini, M., Grillo, S., Esposito, S., 2016. Glucose-6-phosphate dehydrogenase plays a central role in the response of tomato (*Solanum lycopersicum*) plants to short and long-term drought. Plant Physiol. Biochem. 105, 79–89. https://doi.org/10.1016/j.plaphy.2016.04.013

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R., 2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

Massaretto, I.L., Albaladejo, I., Purgatto, E., Flores, F.B., Plasencia, F., Egea-Fernández, J.M., Bolarin, M.C., Egea, I., 2018. Recovering tomato landraces to simultaneously improve fruit yield and nutritional quality against salt stress. Front. Plant Sci. 9, 1778. https://doi.org/10.3389/fpls.2018.01778

Muhammad, I., Shalmani, A., Ali, M., Yang, Q.H., Ahmad, H., Li, F.B., 2021. Mechanisms regulating the dynamics of photosynthesis under abiotic stresses. Front. Plant Sci. 11, 2310. https://doi.org/https://doi.org/10.3389/fpls.2020.615942

Mukhtar, I., Shahid, M.A., Khan, M.W., Balal, R.M., Iqbal, M.M., Naz, T., Zubair, M., Ali, H.H., 2016. Improving salinity tolerance in chili by exogenous application of calcium and sulphur. Soil Environ. 35(1), 56–64

Negrão, S., Schmöckel, S.M., Tester, M., 2017. Evaluating physiological responses of plants to salinity stress. Ann. Bot. 119. https://doi.org/10.1093/aob/mcw191

Nyongesah, M.J., Wang, Q., Li, P., 2015. Effectiveness of photochemical reflectance index to trace vertical and seasonal chlorophyll a/b ratio in *Haloxylon ammodendron*. Acta Physiol. Plant. 37, 2. https://doi.org/10.1007/s11738-014-1747-x

Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60, 324–349. https://doi.org/10.1016/j.ecoenv.2004.06.010

Park, H.J., Kim, W.-Y., Yun, D.-J., 2016. A new insight of salt stress signaling in plant. Mol. Cells 39, 447–59. https://doi.org/10.14348/molcells.2016.0083

Pavlović, I., Pěnčík, A., Novák, O., Vujčić, V., Radić Brkanac, S., Lepeduš, H., Strnad, M., Salopek-Sondi, B., 2018. Short-term salt stress in *Brassica rapa* seedlings causes alterations in auxin metabolism. Plant Physiol. Biochem. 125, 74–84. https://doi.org/10.1016/j.plaphy.2018.01.026

Perri, S., Katul, G.G., Molini, A., 2019. Xylem–phloem hydraulic coupling explains multiple osmoregulatory responses to salt stress. New Phytol. 224, 644–662. https://doi.org/10.1111/nph.16072

Pokotylo, I. V, Kretinin, S. V, Kravets, V.S., 2012. Role of phospholipase D in metabolism reactions of transgenic tobacco cax1 cells under salinity stress. Cytol. Genet. 46, 3–9. https://doi.org/10.3103/S0095452712030085

Rangani, J., Panda, A., Patel, M., Parida, A.K., 2018. Regulation of ROS through proficient modulations of antioxidative defense system maintains the structural and functional integrity of photosynthetic apparatus and confers drought tolerance in the facultative halophyte *Salvadora persica* L. J. Photochem. Photobiol. B Biol. 189, 214–233. https://doi.org/10.1016/j.jphotobiol.2018.10.021

Sahin, U., Ekinci, M., Ors, S., Turan, M., Yildiz, S., Yildirim, E., 2018. Effects of individual and combined effects of salinity and drought on physiological, nutritional and biochemical properties of cabbage (*Brassica oleracea* var. capitata). Sci. Hortic. (Amsterdam). 240, 196–204. https://doi.org/10.1016/j.scienta.2018.06.016

Sarabi, B., Fresneau, C., Ghaderi, N., Bolandnazar, S., Streb, P., Badeck, F.-W., Citerne, S., Tangama, M., David, A., Ghashghaie, J., 2019. Stomatal and non-stomatal limitations are responsible in down-regulation of photosynthesis in melon plants grown under the saline condition: Application of carbon isotope

discrimination as a reliable proxy. Plant Physiol. Biochem. 141, 1–19. https://doi.org/10.1016/j.plaphy.2019.05.010

Shu, S., Guo, S.-R., Sun, J., Yuan, L.-Y., 2012. Effects of salt stress on the structure and function of the photosynthetic apparatus in *Cucumis sativus* and its protection by exogenous putrescine. Physiol. Plant. 146, 285–296. https://doi.org/10.1111/j.1399-3054.2012.01623.x

Srinivas, A., Rajasheker, G., Jawahar, G., Devineni, P.L., Parveda, M., Kumar, S.A., Kavi Kishor, P.B., 2018. Deploying Mechanisms Adapted by Halophytes to Improve Salinity Tolerance in Crop Plants: Focus on Anatomical Features, Stomatal Attributes, and Water Use Efficiency, in: Salinity Responses and Tolerance in Plants, Volume 1. Springer International Publishing, Cham, pp. 41–64. https://doi.org/10.1007/978-3-319-75671-4_2

Stepien, P., Johnson, G.N., 2009. Contrasting responses of photosynthesis to salt stress in the glycophyte Arabidopsis and the halophyte thellungiella: role of the plastid terminal oxidase as an alternative electron sink. Plant Physiol. 149, 1154–65. https://doi.org/10.1104/pp.108.132407

Strasser, R., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples, in: Yunus, M., Pathre, U., Mohanty, P. (Eds.), Probing Photosynthesis: Mechanism, Regulation and Adaptation. Taylor & Francis, London, pp. 443–480

Strasser, R.J., Tsimilli-Michael, M., Srivastava, A., 2004. Analysis of the Chlorophyll a Fluorescence Transient, in: Chlorophyll a Fluorescence. Springer Netherlands, Dordrecht, pp. 321–362. https://doi.org/10.1007/978-1-4020-3218-9_12

Till, B.J., 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res. 13, 524–530. https://doi.org/10.1101/gr.977903

Wendt, U.K., Wenderoth, I., Tegeler, A., von Schaewen, A., 2000. Molecular characterization of a novel glucose-6-phosphate dehydrogenase from potato (*Solanum tuberosum* L.). Plant J. 23, 723–733. https://doi.org/10.1046/j.1365-313x.2000.00840.x

Yang, J., Kong, Q., Xiang, C., 2009. Effects of low night temperature on pigments, chl a fluorescence and energy allocation in two bitter gourd (*Momordica charantia* L.) genotypes. Acta Physiol. Plant. 31, 285–293. https://doi.org/10.1007/s11738-008-0231-x

Zhao, X., Bai, X., Jiang, C., Li, Z., 2019. Phosphoproteomic analysis of two contrasting maize inbred lines provides insights into the mechanism of salt-stress tolerance. Int. J. Mol. Sci. 20, 1886. https://doi.org/10.3390/ijms20081886

Supporting information

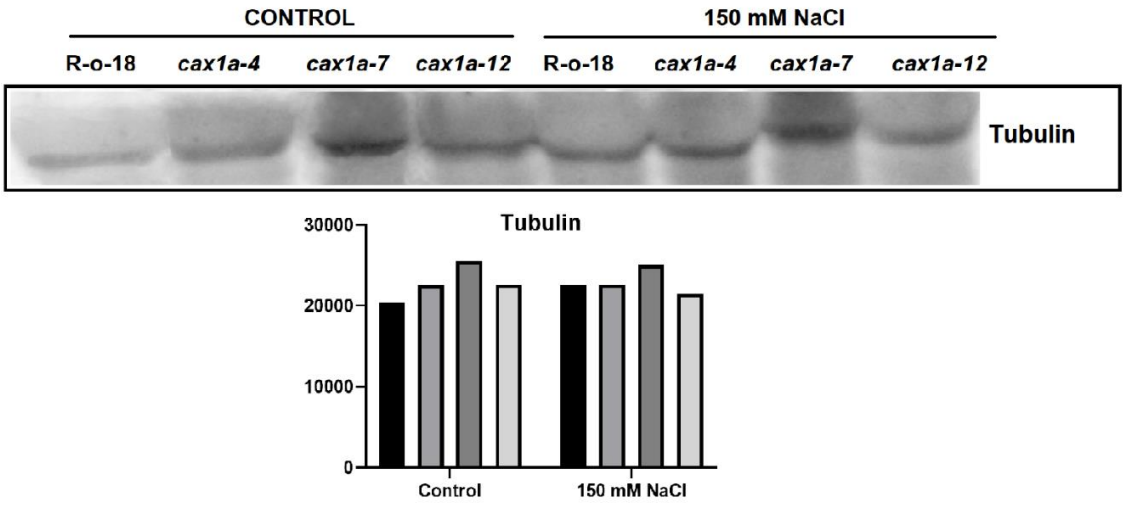
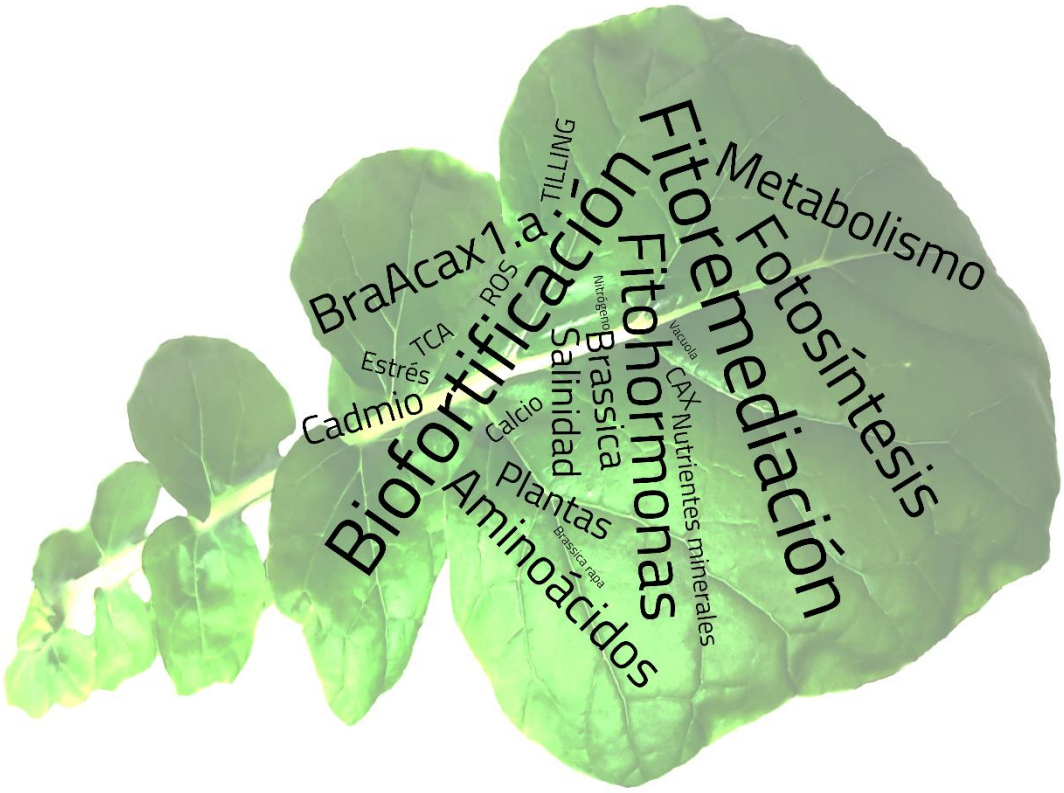


Figure S1. Result of control blot using anti-tubulin antisera. The upper panel shows western blotting. Lower panel shows densitometric analysis using Image J software.

DISCUSIÓN GENERAL



El objetivo de esta sección es proporcionar una visión general de la investigación llevada a cabo durante esta Tesis Doctoral, integrando los distintos resultados obtenidos y discutiendo las perspectivas futuras.

La agricultura necesita de NBTs como el TILLING para generar nuevas variedades que respondan a los problemas que afectan actualmente a los cultivos en todo el mundo (Holme *et al.*, 2019). Esta situación cobra una mayor importancia en el actual escenario de cambio climático que, debido a la disminución de tierras cultivables en condiciones óptimas, puede aumentar la urgencia por la generación de estas nuevas variedades (Gomez-Zavaglia *et al.*, 2020). En la presente Tesis se han abordado tres grandes problemas para la agricultura como son las deficiencias nutricionales, la contaminación por metales pesados y la salinidad. A lo largo de sus capítulos se ha comprobado el efecto que tienen estos tres factores en distintos procesos de la fisiología de *B. rapa* y además se ha analizado la distinta respuesta de las líneas mutantes generados por TILLING para el transportador CAX1.

El primer objetivo de esta tesis doctoral fue comprobar cómo se producía la acumulación de Ca^{2+} en las plantas mutantes crecidas a diferentes concentraciones de este catión en el medio de cultivo y evaluar la calidad nutricional y los efectos fisiológicos de cada mutación en CAX1 en la biofortificación con este elemento. El Ca es un nutriente esencial para la nutrición humana que debemos ingerir en cantidad adecuada para tener un buen estado de salud ya que la deficiencia de este macronutriente aumenta la susceptibilidad

a enfermedades como la osteoporosis. En el año 2011, alrededor de un 51% de la población mundial estaban en alto riesgo de deficiencia de Ca, principalmente en África y Asia (Cormick and Belizán, 2019).

Mediante el desarrollo de programas de biofortificación se puede incrementar la cantidad de determinados nutrientes en las partes comestibles de las plantas y por tanto ayudar a paliar esta deficiencia nutricional en humanos. La biofortificación se pude llevar a cabo asegurando un aporte adecuado de nutrientes a la planta o mediante la obtención de variedades con una mayor eficiencia en la acumulación de los nutrientes mediante las NBTs y otras técnicas como el TILLING (Sharma y Verma, 2019). CAX1 es un gen diana muy adecuado para dichas metodologías de mejora debido a que se ha demostrado exhaustivamente su relación con la acumulación y homeostasis del Ca en la planta (Pittman y Hirschi, 2016).

En el capítulo 1 de la presente tesis, se constató que los distintos mutantes *BraA.cax1a* presentaron diferente respuesta ante cambios en la disponibilidad de Ca en el medio en cuanto a su crecimiento, su fisiología y su acumulación de nutrientes. Las distintas mutaciones no causaron una mayor acumulación de Ca ante condiciones deficientes en el medio, pero si aumentaron su acumulación con un aporte óptimo y tóxico de este catión. Cabe destacar que la línea *BraA.cax1a-12* acumuló más Ca que R-o-18 en condiciones control, pero limitó su acumulación ante elevadas concentraciones evitando su toxicidad. *BraA.cax1a-12* presentó una mayor absorción de Ca y una mejor translocación

a la parte aérea en condiciones control y además mostró una elevada eficiencia en su uso ante condiciones tóxicas (Figura 1). Los efectos de las mutaciones de CAX1 en la acumulación de Ca probablemente se deban a que este transportador es importante para evitar la toxicidad de este elemento ya que promueve su almacenamiento en la vacuola. En este proceso se reducirían los niveles citosólicos de Ca aunque al mismo tiempo se produciría un incremento en la entrada de Ca en las células dando lugar a una mayor absorción de Ca por parte de la planta (Pittman and Hirschi, 2016).

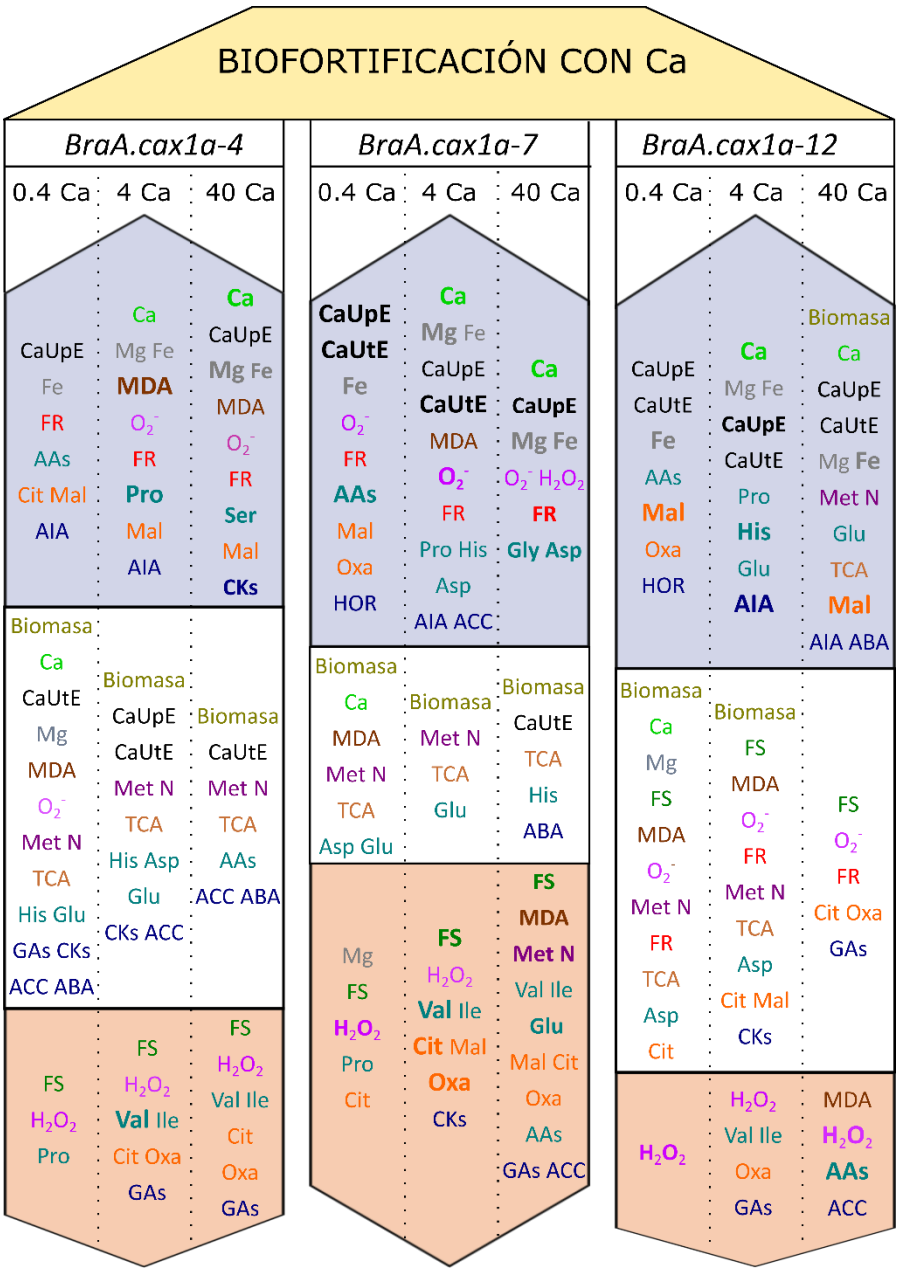


Figura 1. Resumen de los resultados obtenidos en el capítulo 1. La flecha azul hacia arriba engloba los parámetros que incrementan, la flecha roja hacia abajo engloba los parámetros que disminuyen y el espacio central en blanco engloba los parámetros que no varían con respecto a los valores medidos en las plantas R-o-18. Los parámetros destacados en negrita indican que ese genotipo presentó los valores mayores o menores en comparación con el resto de genotipos. Citrato (Cit); Fotosíntesis (FS); Fitohormonas (Hor); Fotorrespiración (FR); Malato (Mal); Metabolismo del N (Met N); Oxalato (Oxa).

En cuanto a los efectos sobre otros elementos, varios estudios realizados en plantas con una expresión de CAX1 modificada informaron de la alteración de las acumulaciones de otros nutrientes minerales aparte del Ca (Hirschi, 1999; Conn *et al.*, 2011). De esta forma, en cuanto a la calidad nutricional, los mutantes empleados en esta Tesis acumularon más Mg en las hojas cuando el Ca se aportó en una concentración adecuada y acumularon más Fe independientemente de la dosis de Ca (Figura 1). Numerosos estudios han constatado que existen relaciones de sinergismo y antagonismos entre los distintos nutrientes minerales en la planta, por lo que el aumento en la absorción de un nutriente puede favorecer o perjudicar a la acumulación de otro (Broadley y White, 2010). Es por ello que el distinto transporte de Ca en los mutantes *BraA.cax1a* puede dar lugar a distinta acumulación de otros nutrientes minerales.

Con respecto a los efectos fisiológicos, los resultados obtenidos muestran que las mutaciones *BraA.cax1a* afectan claramente a la fisiología y al metabolismo de las plantas. Así, *BraA.cax1a-4* y *BraA.cax1a-7* que presentaron alteraciones en la homeostasis del Ca^{2+}, también mostraron síntomas de estrés como una menor cantidad de Chl total, una alteración del rendimiento de la fotosíntesis y mayores niveles de ROS (Figura 1). Numerosos estudios han demostrado que una homeostasis alterada de Ca^{2+} puede producir síntomas de estrés como clorosis y un bajo rendimiento fotosintético (Xiao *et al.*, 2016). Además, los flujos de Ca^{2+} son necesarios en las células guarda para el cierre de los estomas, por lo que la toxicidad de este elemento puede provocar un cierre excesivo de los estomas, la reducción de la concentración interna de CO_2 y una menor tasa fotosíntética, limitando así el crecimiento de las plantas (Dayod *et al.*,

2010). Asimismo, Pokotylo *et al.* (2012) demostraron que una expresión alterada de CAX1 puede conducir a un mayor estrés oxidativo, como se observó en mutantes de tabaco *cax1* que presentaban niveles más altos de MDA y menores actividades de enzimas antioxidantes.

Una adecuada homeostasis de Ca^{2+} también es necesaria para el correcto funcionamiento del metabolismo del N y del TCA al estar implicado en su regulación y en la activación de enzimas clave de estos metabolismos. La mutación *BraA.cax1a-7* redujo la actividad de algunas enzimas del metabolismo N y esta mutación junto con la mutación *BraA.cax1a-4* aumentó la actividad de las enzimas de la fotorrespiración analizadas (Figura 1). Ante un estrés, la fotorrespiración generalmente se activa, produciendo amonio (Zhang and Peng, 2016) y suele prevalecer la degradación de proteínas respecto a su síntesis lo que da lugar a la acumulación de AAs libres especialmente aquellos con una función protectora (Ashraf y Foolad, 2007). Este fenómeno se observó en las plantas *BraA.cax1a-7* que aumentaron la acumulación de AAs de removilización del N. Sin embargo, la mutación *BraA.cax1a-12* no afectó negativamente al metabolismo de la planta y proporcionó una mejor tolerancia a las dosis altas de Ca. Esta tolerancia podría ser proporcionada por la mejora de las actividades de las enzimas del metabolismo del N, de las actividades PEPC y CS, y por una mayor concentración de Glu y malato (Figura 1). Una actividad más alta de las enzimas del TCA es indicativa de una mayor tolerancia al estrés (Bykova y Igamberdiev, 2016). Asimismo, la acumulación de ciertos ácidos orgánicos contribuye a una buena homeostasis celular (Evangelou *et al*., 2007). Por último, los roles de las fitohormonas y del Ca^{2+} en la señalización celular se encuentran

estrechamente relacionados, ya que el Ca^{2+} actúa como señal que puede mediar en una respuesta hormonal adecuada para hacer frente a estreses (Xu y Huang, 2017). La mayor acumulación de AIA y la menor presencia del precursor de etileno también podría contribuir a la tolerancia de *BraA.cax1a-12* (Figura 1). Este hecho se podría explicar debido a que un equilibrio hormonal adecuado con una mayor prevalencia de hormonas promotoras del crecimiento como el AIA frente a hormonas promotoras de la senescencia como el etileno favorece la tolerancia a distintos estreses (Ku *et al.*, 2018).

El segundo objetivo de esta Tesis Doctoral fue comprobar la tolerancia de cada mutante *BraA.cax1a* frente a distintos estreses abióticos y detectar procesos clave que determinen una mayor tolerancia a cada tipo de estrés. El primer estrés analizado fue la toxicidad por Cd ya que la contaminación por este metal pesado es otro de los grandes problemas para la agricultura. El Cd es especialmente peligroso debido a su gran disponibilidad y movilidad dentro de la cadena trófica pasando de un nivel a otro fácilmente y produciendo efectos tóxicos muy importantes. Por ello es preciso controlar la acumulación de este elemento por parte de los cultivos (Clemens y Ma, 2016). Mediante la fitorremediación es posible reducir considerablemente la cantidad de Cd presente en las tierras de cultivo. Para ello se usan especies que sean capaces de crecer en estos suelos y acumulen en una concentración considerable estos metales pesados, preferiblemente en la parte aérea para poder ser fácilmente eliminados (Thakur *et al.*, 2019). Las NBTs han demostrado ser muy útiles para incrementar la eficiencia en la fitorremediación del Cd y modificaciones en el

transportador CAX1 han resultado ser ventajosas para este propósito (Ali-Zade *et al.*, 2010; Baliardini *et al.*, 2015).

Como se constató en el capítulo 2, la mutación *BraA.cax1a-7* y especialmente la mutación *BraA.cax1a-12* proporcionaron una mayor acumulación de Cd en las hojas con respecto a R-o-18. Así, *BraA.cax1a-12* registró la mejor capacidad de fitoextracción con más del triple del contenido de Cd de R-o-18. A pesar de ello, *BraA.cax1a-12* presentó una mayor tolerancia a la toxicidad del Cd, ya que mantuvo un mejor crecimiento que las otras líneas (Figura 2). Esta tolerancia al Cd podría deberse a una mejor homeostasis del Ca^{2+} en condiciones de estrés por este metal. De hecho, se ha demostrado que el Ca, junto con el Mg, son antagonistas del Cd y mitigan sus efectos tóxicos (Zorrig *et al.*, 2010; Tran y Popova, 2014; Srivastava *et al.*, 2015), y en nuestro estudio, todos los mutantes acumularon mayores cantidades de estos elementos, presentándose los valores más altos en las plantas *BraA.cax1a-12,* lo que podría contribuir a su mejor tolerancia al Cd (Figura 2).

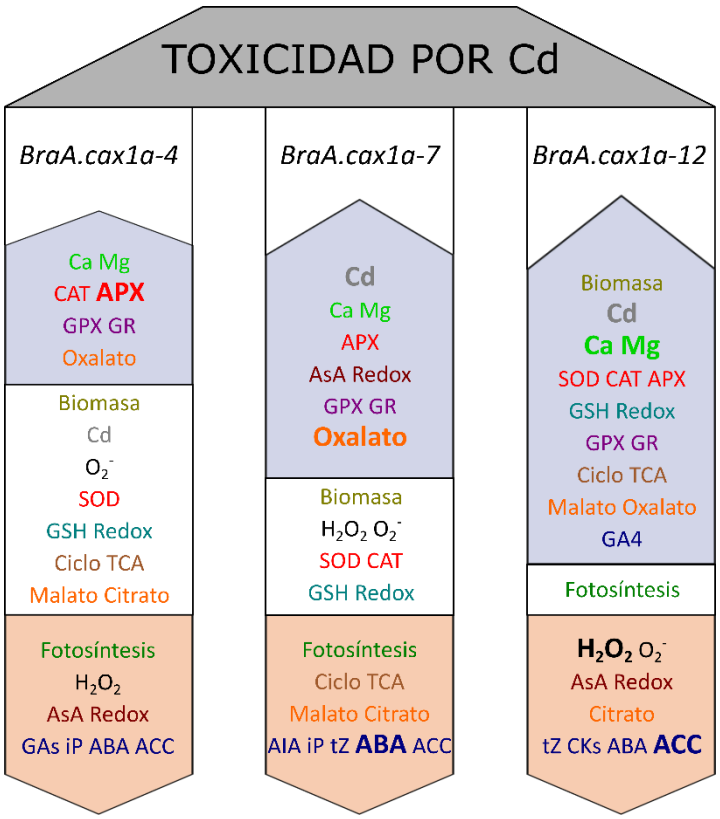


Figura 2. Resumen de los resultados obtenidos en el capítulo 2. La flecha azul hacia arriba engloba los parámetros que incrementan, la flecha roja hacia abajo engloba los parámetros que disminuyen y el espacio central en blanco engloba los parámetros que no varían con respecto a los valores medidos en las plantas R-o-18. Los parámetros destacados en negrita indican que ese genotipo presentó los valores mayores o menores en comparación con el resto de genotipos.

Uno de los efectos más importantes del Cd que afecta al crecimiento de la planta es la inhibición del proceso de fotosíntesis (Clemens y Ma, 2016). Es por ello que, plantas que sean capaces de mantener una adecuada eficiencia fotosintética tendrán una mayor tolerancia al Cd, lo que concuerda con lo observado en los mutantes *BraA.cax1a-12* (Figura 2). Asimismo, una mejor capacidad de detoxificación de ROS a través de compuestos y actividades antioxidantes es clave para una mayor tolerancia al Cd (Sharma *et al*., 2012). En

este sentido, el estudio de Ahmadi *et al.* (2018) demostró que el papel clave en la mejor tolerancia de mutantes CAX1 fue una respuesta antioxidante más eficiente. En la presente Tesis se observó que el genotipo *BraA.cax1a-4* presentó una respuesta similar a la de las plantas R-o-18 aunque tuvo una mayor capacidad de detoxificación de H_2O_2 a través del ciclo AsA/GSH. Sin embargo, *BraA.cax1a-12* presentó la actividad más alta del ciclo de AsA/GSH y una mejor capacidad de detoxificación de ROS (Figura 2). Asimismo, un aumento en la actividad del ciclo TCA y en la producción de OAs que se unen al Cd y mitigan su efecto tóxico dentro de la célula son claves en su tolerancia (Dresler *et al*., 2014; Clemens y Ma, 2016). Este incremento se observó en las plantas *BraA.cax1a-12* con un incremento en la actividad del ciclo TCA y en la acumulación de malato. Por otro lado, el genotipo *BraA.cax1a-7* registró una respuesta negativa porque mostró menores concentraciones de AsA y OAs y actividad más baja del TCA (Figura 2). Por último, la señalización hormonal es clave en la toxicidad por Cd, así como en otros procesos de estrés. Un balance positivo de hormonas promotoras del crecimiento frente a hormonas promotoras del estrés también contribuye a una mayor tolerancia frente al Cd *(*Ahmad *et al*., 2015). De este modo, los niveles más altos de GA4, y la menor presencia del precursor del etileno ACC en plantas *BraA.cax1a-12* también podrían contribuir a su tolerancia frente al Cd (Figura 2).

El segundo tipo de estrés analizado en la presente Tesis Doctoral fue el estrés por salinidad debido a que el aumento en la concentración de sales tanto en las tierras de cultivo como en las aguas de riego es uno de los problemas más importantes para la agricultura que va a ir ganando importancia durante los

próximos años. Así pues, la mayor incidencia de las sequías y el incremento en el nivel del mar en el actual escenario de cambio climático aumentará la presión sobre los cultivos del estrés por salinidad (Acosta-Motos *et al.*, 2017). La sal con una mayor presencia es el NaCl y la acumulación del Na es el principal factor que contribuye a la toxicidad en los cultivos. Sin embargo, puesto que se ha probado que el Ca puede contrarrestar los efectos negativos del Na (Köster *et al.*, 2018), la modificación del transportador CAX1 puede ser positiva en la tolerancia de la salinidad de los cultivos.

En el capítulo 3 se comprobó que las distintas mutaciones *BraA.cax1a* afectaron a la respuesta frente a la salinidad. Así, la mutación *BraA.cax1a-4* proporcionó una mejor tolerancia a la presencia de NaCl en el medio de cultivo. Esta tolerancia podría ser inducida por un mayor contenido de Ca y K en la parte aérea, una eficiente detoxificación de ROS a través de las enzimas SOD y CAT y un mejor estado redox de AsA (Figura 3), ya que se ha comprobado que una adecuada homeostasis del Ca^{2+} y del K^{+} (Wan *et al*., 2017) y una eficaz respuesta antioxidante son claves para contrarrestar el estrés por salinidad (Park *et al*., 2016; García-Martí *et al*., 2019). Además, como respuesta a este tipo de estrés las plantas acumulan compuestos osmocompatibles como Pro y carbohidratos solubles y aumenta la producción de HSP lo que contribuye a mejorar la tolerancia frente a la salinidad (Cantabella *et al*., 2017; Anaraki *et al.*, 2018; Sahin *et al.*, 2018; Chrysargyris *et al*., 2019). En la presente Tesis se observó que la mayor acumulación de Cyt y Chl HSP70 y de Pro observada en las plantas *BraA.cax1a-4* podría contribuir a mejorar la tolerancia de *B. rapa* a la salinidad (Figura 3).

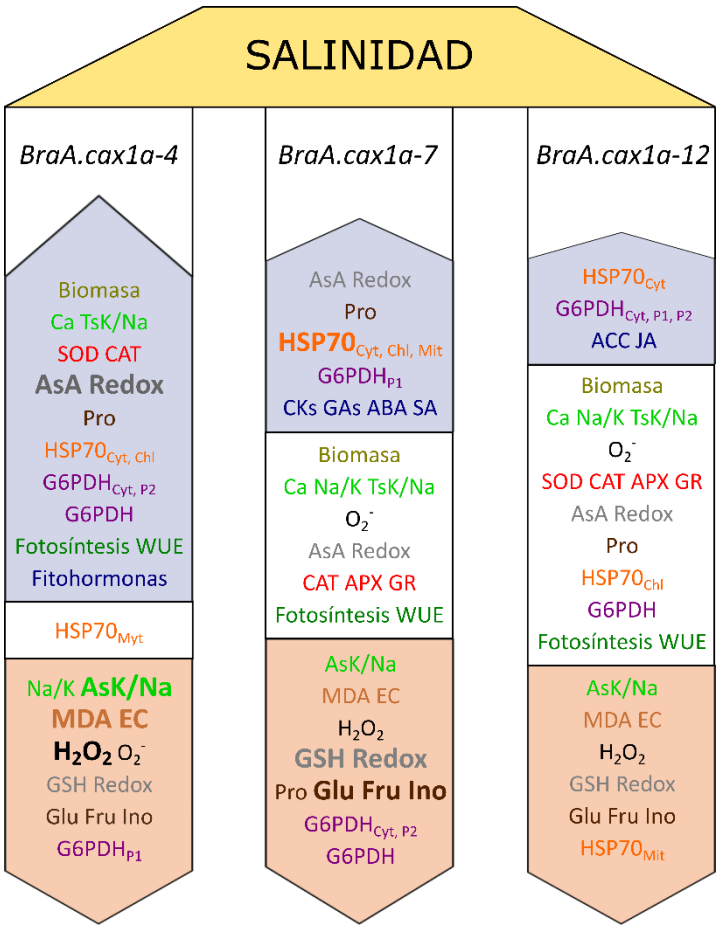


Figura 3. Resumen de los resultados obtenidos en el capítulo 3. La flecha azul hacia arriba engloba los parámetros que incrementan, la flecha roja hacia abajo engloba los parámetros que disminuyen y el espacio central en blanco engloba los parámetros que no varían con respecto a los valores medidos en las plantas R-o-18. Los parámetros destacados en negrita indican que ese genotipo presentó los valores mayores o menores en comparación con el resto de genotipos.

Por otro lado, ante un estrés como la salinidad aumenta la emisión de fluorescencia por parte de la Chl *a* por lo que gran parte de la energía recibida no es aprovechada. Además, se promueve el cierre de estomas para evitar la pérdida de agua y por tanto disminuye el CO_2 disponible para la Rubisco por lo que disminuye su actividad (Parida y Das, 2005). Las plantas que son capaces de mantener el rendimiento fotosintético pueden mantener su crecimiento ante

condiciones de salinidad. A su vez, las plantas con un WUE alto son capaces de regular la apertura estomática de manera que minimicen la pérdida de agua, pero a la vez no se perjudique la asimilación del CO_2 (Ashraf y Harris, 2013). En el capítulo 3 se describió como el estrés por salinidad provocó la reducción en el rendimiento fotosintético en las plantas de *B. rapa*, sin embargo, la mutación B*raA.cax1a-4* proporcionó una mejora en dicho rendimiento que se manifestó en unos valores más altos de WUE, Fv/Fm, flujos de electrones y una mayor acumulación de Rubisco. En el lado opuesto, la mutación *BraA.cax1a-7* produjo algunos efectos negativos en el rendimiento de la fotosíntesis, como por ejemplo valores superiores de pico K y DIo/RC y menores acumulaciones de Rubisco y carbohidratos solubles (Figura 3).

Otro elemento importante en la respuesta a la salinidad es la vía de las pentosas fosfato que es regulada por la enzima G6PDH y es clave en el suministro de poder reductor para los sistemas antioxidantes y energéticos de la célula (Esposito, 2016; Landi *et al.*, 2016). Se ha observado que una actividad G6PDH más alta incrementa la actividad ATPasa de membrana lo que facilita una mayor absorción de K^+ frente al Na^+ y por tanto reduce el ratio Na^+/K^+ (Yang *et al.*, 2019). De esta manera, en el capítulo 3 se describió que las plantas *BraA.cax1a-4* tenían una actividad G6PDH más elevada que podría contribuir a su inferior ratio Na^+/K^+ lo que es positivo en su tolerancia a la salinidad. En contraste, el genotipo *BraA.cax1a-7* presentó una menor acumulación y actividad de G6PDH (Figura 3), lo que limitaría su producción de poder reductor.

Por último, el papel de las fitohormonas también es clave en la respuesta a la salinidad. El ABA tiene especial relevancia ya que regula la expresión de genes de respuesta a estrés y la apertura estomática. Asimismo, unos menores niveles de etileno y mayores de hormonas promotoras del crecimiento pueden mejorar la tolerancia a la salinidad (Acosta-Motos *et al.*, 2017). En el capítulo 3 se describió que, como respuesta a la salinidad, todas las plantas aumentaron los niveles de ABA, ACC y SA, pero se encontraron diferencias en el perfil hormonal de los genotipos estudiados. Destacó una mayor acumulación de ACC en todos los mutantes *BraA.cax1a* y, específicamente *BraA.cax1a-4* mostró una concentración más alta de fitohormonas que podría estar relacionada con su mejor tolerancia a la sal. Concretamente, las mayores concentraciones de AIA y GAs podrían promover el crecimiento en condiciones salinas y el ABA podría dar lugar a una mejor respuesta frente al estrés y a una mejor eficiencia en el uso del agua. Además, los genes G6PDH presentan elementos de respuesta al ABA (Cardi *et al.*, 2011) por lo que la concentración más elevada de ABA en las plantas *BraA.cax1a-4* también puede contribuir a la mayor acumulación de G6PDH observada en estas plantas (Figura 3).

En esta Tesis Doctoral se ha puesto de manifiesto el importante papel de CAX1 dentro de la planta ya que variaciones en su secuencia aminoacídica conllevan importantes cambios fisiológicos. Así, como se ha indicado a lo largo de la presente memoria, las distintas mutaciones en CAX1a producidas por TILLING tienen distintos efectos en la fisiología de *B. rapa* y ante las distintas condiciones de cultivo realizadas. Destaca la mutación *BraA.cax1a-12* que sería útil para la biofortificación nutricional y para la fitorremediación de suelos

contaminados por Cd y a la mutación *BraA.cax1a-4* que puede contribuir a una mayor tolerancia frente a la salinidad. Por otro lado, se ha constatado que la mutación *BraA.cax1a-7* produce alteraciones en diversos parámetros fisiológicos por lo que se confirma la necesidad de un correcto funcionamiento del transportador CAX1 para un normal desempeño de los procesos fisiológicos de la planta.

Este trabajo no da respuesta al mecanismo molecular por el cual se producen los efectos de cada mutación. Por tanto, sería de gran interés el estudio de dichos mecanismos para conocer el efecto concreto de cada mutación en la estructura y el funcionamiento del transportador CAX1a de *B. rapa* y poder replicar dicho efecto en otras especies de interés agronómico. Asimismo, también sería interesante ensayar el comportamiento de los mutantes *BraA.cax1a* en condiciones de campo y llevar su cultivo hasta cosecha, para comprobar si dichas mutaciones afectan a aspectos claves como son el rendimiento, la producción y la viabilidad de sus semillas. Igualmente, sería interesante replicar las mismas mutaciones en otras especies de la familia Brassicaceae con gran interés económico y comercial como son las distintas variedades comerciales de *B. oleracea* entre las que se incluyen la col, la coliflor y el brócoli. De este modo, las mutaciones empleadas en la presente Tesis serían muy interesantes para incrementar la calidad nutricional de dichos cultivos y aumentar la tolerancia a estreses de importancia creciente en los próximos años como es la salinidad.

CONCLUSIONES / CONCLUSIONS

1. Las mutaciones en el transportador CAX1a causaron cambios en el crecimiento, metabolismo, estado fisiológico y la acumulación de nutrientes en las plantas de *B. rapa* y estos cambios se vieron influidos por las dosis de Ca aplicadas. *BraA.cax1a-4* y especialmente *BraA.cax1a-7* mostraron síntomas de estrés que fisiológicamente se vio reflejado en un menor rendimiento fotosintético y mayores niveles de ROS, una alteración del metabolismo del N y una activación de la fotorrespiración. Por otro lado, la mutación *BraA.cax1a-12* permitió un mejor crecimiento en condiciones de alto contenido de Ca y proporcionó una mejora de varios procesos metabólicos estudiados. Además, las tres mutaciones podrían ser útiles para el desarrollo de programas de biofortificación con Ca y Mg cuando el Ca es aplicado en una concentración adecuada en el medio, y para la biofortificación con Fe utilizando cualquiera de la dosis de Ca aplicadas en este estudio.

2. La mutación *BraA.cax1a-12* podría ser útil en la fitorremediación de suelos contaminados con Cd ya que proporcionó un considerable aumento en la acumulación de este metal pesado, pero sin efectos negativos en el crecimiento. Además, los parámetros fisiológicos analizados mostraron una respuesta negativa del genotipo *BraA.cax1a-7* mientras que dicha respuesta fue positiva en el genotipo *BraA.cax1a-12* destacando una mayor detoxificación de ROS y un mejor rendimiento fotosintético.

3. La mutación *BraA.cax1a-4* podría proporcionar una mayor tolerancia al estrés por salinidad. Esta tolerancia podría ser favorecida por la

optimización del balance entre el Na^+ y el K^+, por una mejor respuesta antioxidante y fitohormonal, una mayor generación de poder reductor, y un mejor rendimiento fotosintético y eficiencia en el uso del agua. Sin embargo, la mutación *BraA.cax1a-7* produjo algunos efectos negativos en la fisiología de las plantas mientras que el genotipo *BraA.cax1a-12* no causó efectos destacables en las plantas crecidas en condiciones de salinidad.

CONCLUSIONS

1. Mutations in the CAX1a transporter caused changes in growth, metabolism, physiological state, and nutrient accumulation in *B. rapa* plants and these changes were influenced by the Ca supply. *BraA.cax1a-4* and especially *BraA.cax1a-7* showed stress symptoms which physiologically were reflected in lower photosynthetic performance, higher ROS levels, altered N metabolism, and activation of photorespiration. On the other hand, the *BraA.cax1a-12* mutation allowed better growth under high Ca conditions and provided an improvement of several metabolic processes. In addition, all three mutations could be useful for the development of Ca and Mg biofortification programs, as long as Ca is applied at an adequate concentration in the medium, and for Fe biofortification using any of the Ca doses applied in this study.

2. The *BraA.cax1a-12* mutation could be useful in phytoremediation of Cd-contaminated soils because it provided a considerable increase in the accumulation of this heavy metal, but without negative effects on growth. Moreover, the physiological parameters analyzed showed a negative response of the *BraA.cax1a-7* genotype whereas such response was positive in the *BraA.cax1a-12* genotype, highlighting a higher ROS detoxification and a better photosynthetic performance.

3. The *BraA.cax1a-4* mutation could provide greater tolerance to salinity stress. This tolerance could be favored by the optimization of Na^+ and K^+

balance, enhanced antioxidant and phytohormonal response, increased reducing power generation, and improved photosynthetic performance and water use efficiency. However, the *BraA.cax1a-7* mutation produced some negative effects on plant physiology while the *BraA.cax1a-12* genotype caused no remarkable effects on plants grown under salinity conditions.

BIBLIOGRAFÍA GENERAL

Acosta-Motos, J., Ortuño, M., Bernal-Vicente, A., Diaz-Vivancos, P., Sanchez-Blanco, M., Hernandez, J., 2017. Plant responses to salt stress: Adaptive mechanisms. Agronomy 7, 18. https://doi.org/10.3390/agronomy7010018

Ahmad, A., Hadi, F., Ali, N., 2015. Effective phytoextraction of cadmium (Cd) with increasing concentration of total phenolics and free proline in *Cannabis sativa* (L) plant under various treatments of fertilizers, plant growth regulators and sodium salt. Int. J. Phytoremediation 17, 56–65. https://doi.org/10.1080/15226514.2013.828018

Ahmadi, H., Corso, M., Weber, M., Verbruggen, N., Clemens, S., 2018. CAX1 suppresses Cd-induced generation of reactive oxygen species in *Arabidopsis halleri*. Plant. Cell Environ. 41, 2435–2448. https://doi.org/10.1111/pce.13362

Ashraf, M., Foolad, M.R., 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 59, 206–216. https://doi.org/10.1016/j.envexpbot.2005.12.006

Ashraf, M., Harris, P.J.C., 2013. Photosynthesis under stressful environments: An overview. Photosynthetica 51, 163–190. https://doi.org/10.1007/s11099-013-0021-6

Ali-Zade, V., Alirzayeva, E., Shirvani, T., 2010. Plant Resistance to Anthropogenic Toxicants: Approaches to Phytoremediation, in: Plant Adaptation and Phytoremediation. Springer Netherlands, Dordrecht, pp. 173–192. https://doi.org/10.1007/978-90-481-9370-7_9

Anaraki, Z.E., Tafreshi, S.A.H., Shariati, M., 2018. Transient silencing of heat shock proteins showed remarkable roles for HSP70 during adaptation to stress in plants. Environ. Exp. Bot. 155, 142–157. https://doi.org/10.1016/j.envexpbot.2018.06.031

Anwar, A., Kim, J.-K., 2020. Transgenic Breeding Approaches for Improving Abiotic Stress Tolerance: Recent Progress and Future Perspectives. Int. J. Mol. Sci. 21, 2695. https://doi.org/10.3390/ijms21082695

Atif, R.M., Shahid, L., Waqas, M., Ali, B., Rashid, M.A.R., Azeem, F., Nawaz, M.A., Wani, S.H., Chung, G., 2019. Insights on Calcium-Dependent Protein Kinases (CPKs) Signaling for Abiotic Stress Tolerance in Plants. Int. J. Mol. Sci. 20. https://doi.org/10.3390/ijms20215298

Azevedo, R.A., Gratão, P.L., Monteiro, C.C., Carvalho, R.F., 2012. What is new in the research on cadmium-induced stress in plants? Food Energy Secur. 1, 133–140. https://doi.org/10.1002/fes3.10

Baethgen, W.E., Alley, M.M., 1989. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digests. Commun. Soil Sci. Plant Anal. 20, 961–969. https://doi.org/10.1080/00103628909368129

Baker, A., McGrath, S., Reeves, R., Smith, J., 2000. Metal Hyperaccumulator Plants: A Review of the Ecology and Physiology of a Biological Resource for Phytoremediation of Metal-Polluted Soils, in: Terry, N., Bañuelos, G. (Eds.), Phytoremediation of Contaminated Soil and Water. Lewis Publisher, pp. 85–107

Baliardini, C., Meyer, C.-L., Salis, P., Saumitou-Laprade, P., Verbruggen, N., 2015. CATION EXCHANGER1 cosegregates with cadmium tolerance in the metal hyperaccumulator *Arabidopsis halleri* and plays a role in limiting oxidative stress in *Arabidopsis* Spp. Plant Physiol. 169, 549–59. https://doi.org/10.1104/pp.15.01037

Beatty, P.H., Wong, J.L., 2017. Nitrogen Use Efficiency. Encycl. Appl. Plant Sci. 278–284. https://doi.org/10.1016/b978-0-12-394807-6.00138-6

Blasco, B., Graham, N.S., Broadley, M.R., 2015. Antioxidant response and carboxylate metabolism in Brassica rapa exposed to different external Zn, Ca, and Mg supply. J. Plant Physiol. 176, 16–24. https://doi.org/10.1016/j.jplph.2014.07.029

Bradshaw, H.D., 2005. Mutations in CAX1 produce phenotypes characteristic of plants tolerant to serpentine soils. New Phytol. 167, 81–88. https://doi.org/10.1111/j.1469-8137.2005.01408.x

Broadley, M.R., White, P.J., 2010. Eats roots and leaves. Can edible horticultural crops address dietary calcium, magnesium and potassium deficiencies? Proc. Nutr. Soc. 69, 601–12. https://doi.org/10.1017/S0029665110001588

Bykova, N. V., Igamberdiev, A.U., 2016. Redox State in Plant Mitochondria and its Role in Stress Tolerance, in: Redox State as a Central Regulator of Plant-Cell Stress Responses. Springer International Publishing, Cham, pp. 93–115. https://doi.org/10.1007/978-3-319-44081-1_5

Cantabella, D., Piqueras, A., Acosta-Motos, J.R., Bernal-Vicente, A., Hernández, J.A., Díaz-Vivancos, P., 2017. Salt-tolerance mechanisms induced in *Stevia rebaudiana* Bertoni: Effects on mineral nutrition, antioxidative metabolism and

steviol glycoside content. Plant Physiol. Biochem. 115, 484–496. https://doi.org/10.1016/J.PLAPHY.2017.04.023

Cardi, M., Chibani, K., Cafasso, D., Rouhier, N., Jacquot, J.-P., Esposito, S., 2011. Abscisic acid effects on activity and expression of barley (*Hordeum vulgare*) plastidial glucose-6-phosphate dehydrogenase. J. Exp. Bot. 62, 4013–4023. https://doi.org/10.1093/jxb/err100

Catalá, R., Santos, E., Alonso, J.M., Ecker, J.R., Martínez-Zapater, J.M., Salinas, J., 2003. Mutations in the Ca^{2+}/H^{+} transporter CAX1 increase CBF/DREB1 expression and the cold-acclimation response in *Arabidopsis*. Plant Cell 15, 2940–2951. https://doi.org/10.1105/tpc.015248

Chakradhar, T., Mahanty, S., Reddy, R.A., Divya, K., Reddy, P.S., Reddy, M.K., 2017. Biotechnological Perspective of Reactive Oxygen Species (ROS)-Mediated Stress Tolerance in Plants, in: Reactive Oxygen Species and Antioxidant Systems in Plants: Role and Regulation under Abiotic Stress. Springer Singapore, Singapore, pp. 53–87. https://doi.org/10.1007/978-981-10-5254-5_3

Chao, L., Bofu, P., Weiqian, C., Yun, L., Hao, H., Liang, C., Xiaoqing, L., Xiao, W., Fashui, H., 2008. Influences of calcium deficiency and cerium on growth of spinach plants. Biol. Trace Elem. Res. 121, 266–275. https://doi.org/10.1007/s12011-007-8054-y

Cheng, N.-H., Pittman, J.K., Shigaki, T., Lachmansingh, J., LeClere, S., Lahner, B., Salt, D.E., Hirschi, K.D., 2005. Functional association of Arabidopsis CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol. 138, 2048–2060. https://doi.org/10.1104/pp.105.061218

Cheng, N.H., Pittman, J.K., Barkla, B.J., Shigaki, T., Hirschi, K.D., 2003. The Arabidopsis cax1 mutant exhibits impaired ion homeostasis, development, and hormonal responses and reveals interplay among vacuolar transporters. Plant Cell 15, 347–364. https://doi.org/10.1105/tpc.007385.348

Cho, D., Villiers, F., Kroniewicz, L., Lee, S., Seo, Y.J., Hirschi, K.D., Leonhardt, N., Kwak, J.M., 2012. Vacuolar CAX1 and CAX3 influence auxin transport in guard cells via regulation of apoplastic pH. Plant Physiol. 160, 1293–302. https://doi.org/10.1104/pp.112.201442

Cho, S.-C., Chao, Y.-Y., Kao, C.H., 2012. Calcium deficiency increases Cd toxicity and Ca is required for heat-shock induced Cd tolerance in rice seedlings. J. Plant Physiol. 169, 892–898. https://doi.org/10.1016/j.jplph.2012.02.005

Chrysargyris, A., Solomou, M., Petropoulos, S.A., Tzortzakis, N., 2019. Physiological and biochemical attributes of *Mentha spicata* when subjected to saline conditions and cation foliar application. J. Plant Physiol. 232, 27–38. https://doi.org/10.1016/j.jplph.2018.10.024

Clemens, S., Ma, J.F., 2016. Toxic heavy metal and metalloid accumulation in crop plants and foods. Annu. Rev. Plant Biol. 67, 489–512. https://doi.org/10.1146/annurev-arplant-043015-112301

Conn, S.J., Gilliham, M., Athman, A., Schreiber, A.W., Baumann, U., Moller, I., Cheng, N.-H., Stancombe, M.A., Hirschi, K.D., Webb, A.A.R., Burton, R., Kaiser, B.N., Tyerman, S.D., Leigh, R.A., 2011. Cell-specific vacuolar calcium storage mediated by CAX1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis*. Plant Cell 23, 240–257. https://doi.org/10.1105/tpc.109.072769

Cormick, G., Belizán, J.M., 2019. Calcium Intake and Health. Nutrients 11, 1606. https://doi.org/10.3390/nu11071606

Court-of-Justice-of-the-European-Union, 2018. Organisms obtained by mutagenesis are GMOs and are, in principle, subject to the obligations laid down by the GMO Directive

D'Alessandro, A., Taamalli, M., Gevi, F., Timperio, A.M., Zolla, L., Ghnaya, T., 2013. Cadmium stress responses in Brassica juncea : Hints from proteomics and metabolomics. J. Proteome Res. 12, 4979–4997. https://doi.org/10.1021/pr400793e

Dayod, M., Tyerman, S.D., Leigh, R.A., Gilliham, M., 2010. Calcium storage in plants and the implications for calcium biofortification. Protoplasma 247, 215–231. https://doi.org/10.1007/s00709-010-0182-0

de Freitas, S.T., McElrone, A.J., Shackel, K.A., Mitcham, E.J., 2013. Calcium partitioning and allocation and blossom-end rot development in tomato plants in response to whole-plant and fruit-specific abscisic acid treatments. J. Exp. Bot. 65, 235–247

Dresler, S., Hanaka, A., Bednarek, W., Maksymiec, W., 2014. Accumulation of low-molecular-weight organic acids in roots and leaf segments of Zea mays plants treated with cadmium and copper. Acta Physiol. Plant. 36, 1565–1575. https://doi.org/10.1007/s11738-014-1532-x

Esposito, S., 2016. Nitrogen Assimilation, Abiotic Stress and Glucose 6-Phosphate Dehydrogenase: The Full Circle of Reductants. Plants 5, 24. https://doi.org/10.3390/plants5020024

Evangelou, M.W.H., Ebel, M., Schaeffer, A., 2007. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. Chemosphere 68, 989–1003. https://doi.org/10.1016/j.chemosphere.2007.01.062

Ferreira, K.N., Iverson, T.M., Maghlaoui, K., Barber, J., Iwata, S., 2004. Architecture of the photosynthetic oxygen-evolving center. Science 303(5665), 1831-1838

Francisco, M., Tortosa, M., Martínez-Ballesta, M. de. C., Velasco, P., García-Viguera, C., Moreno, D.A., 2017. Nutritional and phytochemical value of Brassica crops from the agri-food perspective. Ann. Appl. Biol. 170, 273–285. https://doi.org/10.1111/aab.12318

Galili, G., Amir, R., Fernie, A.R., 2016. The regulation of essential amino acid synthesis and accumulation in plants. Annu. Rev. Plant Biol. 67, 153–178. https://doi.org/10.1146/annurev-arplant-043015-112213

Gao, H., Jia, Y., Guo, S., Lv, G., Wang, T., Juan, L., 2011. Exogenous calcium affects nitrogen metabolism in root-zone hypoxia-stressed muskmelon roots and enhances short-term hypoxia tolerance. J. Plant Physiol. 168, 1217–1225. https://doi.org/10.1016/j.jplph.2011.01.022

Gao, H., Wu, X., Zorrilla, C., Vega, S.E., Palta, J.P., 2019. Fractionating of calcium in tuber and leaf tissues explains the calcium deficiency symptoms in potato plant overexpressing CAX1. Front. Plant Sci. 10. https://doi.org/10.3389/fpls.2019.01793

García-Martí, M., Piñero, M.C., García-Sanchez, F., Mestre, T.C., López-Delacalle, M., Martínez, V., Rivero, R.M., 2019. Amelioration of the oxidative stress generated by simple or combined abiotic stress through the K^+ and Ca^{2+} supplementation in tomato plants. Antioxidants 8, 81. https://doi.org/10.3390/antiox8040081

Gill, S.., Tujeta, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Gilroy, S., Suzuki, N., Miller, G., Choi, W.G., Toyota, M., Devireddy, A.R., Mittler, R., 2014. A tidal wave of signals: Calcium and ROS at the forefront of rapid systemic signaling. Trends Plant Sci.. https://doi.org/10.1016/j.tplants.2014.06.013

Gomez-Zavaglia, A., Mejuto, J.C., Simal-Gandara, J., 2020. Mitigation of emerging implications of climate change on food production systems. Food Res. Int. 134, 109256. https://doi.org/10.1016/j.foodres.2020.109256

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of Brassica under altered Ca supply identifies Arabidopsis Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Gururani, M.A., Venkatesh, J., Tran, L.S.P., 2015. Regulation of photosynthesis during abiotic stress-induced photoinhibition. Mol. Plant 8, 1304–1320. https://doi.org/10.1016/j.molp.2015.05.005

Han, N., Shao, Q., Bao, H., Wang, B., 2011. Cloning and characterization of a Ca^{2+}/H^{+} antiporter from halophyte *Suaeda salsa* L. Plant Mol. Biol. Report. 29, 449–457. https://doi.org/10.1007/s11105-010-0244-7

Hirschi, K., 2001. Vacuolar H^{+}/Ca^{2+} transport: who's directing the traffic? Trends Plant Sci. 6, 100–104. https://doi.org/10.1016/s1360-1385(00)01863-x

Hirschi, K.D., 1999. Expression of Arabidopsis CAX1 in tobacco: altered calcium homeostasis and increased stress sensitivity. Plant Cell 11, 2113–2122. https://doi.org/10.1105/tpc.11.11.2113

Hochmal, A.K., Schulze, S., Trompelt, K., Hippler, M., 2015. Calcium-dependent regulation of photosynthesis. Biochim. Biophys. Acta - Bioenerg. 1847, 993–1003. https://doi.org/10.1016/j.bbabio.2015.02.010

Holme, I.B., Gregersen, P.L., Brinch-Pedersen, H., 2019. Induced Genetic Variation in Crop Plants by Random or Targeted Mutagenesis: Convergence and Differences. Front. Plant Sci. 10, 1468. https://doi.org/10.3389/fpls.2019.01468

Huang, D., Gong, X., Liu, Y., Zeng, G., Lai, C., Bashir, H., Zhou, L., Wang, D., Xu, P., Cheng, M., Wan, J., 2017. Effects of calcium at toxic concentrations of cadmium in plants. Planta 245, 863–873. https://doi.org/10.1007/s00425-017-2664-1

Hunter, M.C., Smith, R.G., Schipanski, M.E., Atwood, L.W., Mortensen, D.A., 2017. Agriculture in 2050: Recalibrating Targets for Sustainable Intensification. Bioscience 67, 386–391. https://doi.org/10.1093/biosci/bix010

Igamberdiev, A.U., Eprintsev, A.T., 2016. Organic acids: The pools of fixed carbon involved in redox regulation and energy balance in higher plants. Front. Plant Sci. 7, 1042. https://doi.org/10.3389/fpls.2016.01042

Köster, P., Wallrad, L., Edel, K.H., Faisal, M., Alatar, A.A., Kudla, J., 2018. The battle of two ions: Ca^{2+} signalling against Na^{+} stress. Plant Biol. 21, 39–48. https://doi.org/10.1111/plb.12704

Krouk, G., 2017. Nitrate signalling: Calcium bridges the nitrate gap. Nat. Plants 3, 17095. https://doi.org/10.1038/nplants.2017.95

Ku, Y.-S., Sintaha, M., Cheung, M.-Y., Lam, H.-M., 2018. Plant hormone signaling crosstalks between biotic and abiotic stress responses. Int. J. Mol. Sci. 19, 3206. https://doi.org/10.3390/ijms19103206

Landi, S., Nurcato, R., De Lillo, A., Lentini, M., Grillo, S., Esposito, S., 2016. Glucose-6-phosphate dehydrogenase plays a central role in the response of tomato (*Solanum lycopersicum*) plants to short and long-term drought. Plant Physiol. Biochem. 105, 79–89. https://doi.org/10.1016/j.plaphy.2016.04.013

Lee, J., Park, I., Lee, Z.-W., Kim, S.W., Baek, N., Park, H.-S., Park, S.U., Kwon, S., Kim, H., 2013. Regulation of the major vacuolar Ca^{2+} transporter genes, by intercellular Ca^{2+} concentration and abiotic stresses, in tip-burn resistant *Brassica oleracea*. Mol. Biol. Rep. 40, 177–188. https://doi.org/10.1007/s11033-012-2047-4

Li, H., Pu, P., Li, X., Gong, Y., An, D., Zhang, L., Lv, J., 2020. Sulfur application reduces cadmium uptake in edible parts of pakchoi (*Brassica chinensis* L.) by cadmium chelation and vacuolar sequestration. Ecotoxicol. Environ. Saf. 194, 110402. https://doi.org/10.1016/j.ecoenv.2020.110402

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R., 2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS

mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

Mahmud, J. Al, Hasanuzzaman, M., Nahar, K., Bhuyan, M.H.M.B., Fujita, M., 2018. Insights into citric acid-induced cadmium tolerance and phytoremediation in *Brassica juncea* L.: Coordinated functions of metal chelation, antioxidant defense and glyoxalase systems. Ecotoxicol. Environ. Saf. 147, 990–1001. https://doi.org/10.1016/j.ecoenv.2017.09.045

Manohar, M., Shigaki, T., Hirschi, K.D., 2011. Plant cation/H^+ exchangers (CAXs): Biological functions and genetic manipulations. Plant Biol. 13, 561–569. https://doi.org/10.1111/j.1438-8677.2011.00466.x

Marschner, H., 2012Mineral Nutrition of Higher Plants. London

Mei, H., Zhao, J., Pittman, J.K., Lachmansingh, J., Park, S., Hirschi, K.D., 2007. In planta regulation of the Arabidopsis Ca^{2+}/H^+ antiporter CAX1. J. Exp. Bot. 58, 3419–3427. https://doi.org/10.1093/jxb/erm190

Morris, J., Hawthorne, K.M., Hotze, T., Abrams, S. a, Hirschi, K.D., 2008. Nutritional impact of elevated calcium transport activity in carrots. Proc. Natl. Acad. Sci. U. S. A. 105, 1431–5. https://doi.org/10.1073/pnas.0709005105

Negrão, S., Schmöckel, S.M., Tester, M., 2017. Evaluating physiological responses of plants to salinity stress. Ann. Bot. 119. https://doi.org/10.1093/aob/mcw191

Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60, 324–349. https://doi.org/10.1016/j.ecoenv.2004.06.010

Park, H.J., Kim, W.-Y., Yun, D.-J., 2016. A new insight of salt stress signaling in plant. Mol. Cells 39, 447–59. https://doi.org/10.14348/molcells.2016.0083

Park, S., Elless, M.P., Park, J., Jenkins, A., Lim, W., Chambers IV, E., Hirschi, K.D., 2009. Sensory analysis of calcium-biofortified lettuce. Plant Biotechnol. J. 7, 106–117. https://doi.org/10.1111/j.1467-7652.2008.00379.x

Park, Sunghun, Kang, T.S., Kim, C.K., Han, J.S., Kim, S., Smith, R.H., Pike, L.M., Hirschi, K.D., 2005. Genetic manipulation for enhancing calcium content in potato tuber. J. Agric. Food Chem. 53, 5598–5603. https://doi.org/10.1021/jf050531c

Park, S, NH, C., JK, P., KS, Y., Park, J., RH, S., KD, H., 2005. Increased calcium levels and prolonged shelf life in tomatoes expressing Arabidopsis H^+/Ca^{2+} transporters. Plant Physiol 139(3), 1194–1206. https://doi.org/10.1104/pp.105.066266

Pittman, J.K., Hirschi, K.D., 2016. CAX-ing a wide net: Cation/H^+ transporters in metal remediation and abiotic stress signaling. Plant Biol. 18, 741–749. https://doi.org/10.1111/plb.12460

Pokotylo, I. V., Kretinin, S. V., Kravets, V.S., 2012. Role of phospholipase D in metabolic reactions of transgenic tobacco cax1 cells under the influence of salt stress. Cytol. Genet. 46, 131–135. https://doi.org/10.3103/s0095452712030085

Rana, D., Boogaart, T., O'Neill, C.M., Hynes, L., Bent, E., Macpherson, L., Park, J.Y., Lim, Y.P., Bancroft, I., 2004. Conservation of the microstructure of genome segments in *Brassica napus* and its diploid relatives. Plant J. 40, 725–733. https://doi.org/10.1111/j.1365-313x.2004.02244.x

Rangani, J., Panda, A., Patel, M., Parida, A.K., 2018. Regulation of ROS through proficient modulations of antioxidative defense system maintains the structural and functional integrity of photosynthetic apparatus and confers drought tolerance in the facultative halophyte Salvadora persica L. J. Photochem. Photobiol. B Biol. 189, 214–233. https://doi.org/10.1016/j.jphotobiol.2018.10.021

Rios, J.J., Lochlainn, S.O., Devonshire, J., Graham, N.S., Hammond, J.P., King, G.J., White, P.J., Kurup, S., Broadley, M.R., 2012. Distribution of calcium (Ca) and magnesium (Mg) in the leaves of *Brassica rapa* under varying exogenous Ca and Mg supply. Ann. Bot. 109, 1081–1089. https://doi.org/10.1093/aob/mcs029

Rodríguez-Serrano, M., Romero-Puertas, M.C., Pazmiño, D.M., Testillano, P.S., Risueño, M.C., Del Río, L.A., Sandalio, L.M., 2009. Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. Plant Physiol. 150, 229–43. https://doi.org/10.1104/pp.108.131524

Romero-Marco, P., Pérez-Gallardo, L.L., 2020. Adequacy of calcium intake in Spanish population according age groups. Arch. Osteoporos. 15, 161. https://doi.org/10.1007/s11657-020-00831-3

Ruiz Galán, O., Rodríguez, J.P., 2013. Impacto de la biotecnología en los sectores agrícuola y ganadero 2025, in: Informe de Prospectiva Tecnológica. Madrid: Fundación Española Para La Ciencia y La Tecnología, FECYT

Sahin, U., Ekinci, M., Ors, S., Turan, M., Yildiz, S., Yildirim, E., 2018. Effects of individual and combined effects of salinity and drought on physiological, nutritional and biochemical properties of cabbage (*Brassica oleracea* var. capitata). Sci. Hortic. (Amsterdam). 240, 196–204. https://doi.org/10.1016/j.scienta.2018.06.016

Sandalio, L.M., Rodríguez-Serrano, M., del Río, L.A., Romero-Puertas, M.C., 2009. Reactive Oxygen Species and Signaling in Cadmium Toxicity. Springer, Berlin, Heidelberg, pp. 175–189. https://doi.org/10.1007/978-3-642-00390-5_11

Sanders, D., Pelloux, J., Brownlee, C., Harper, J.F., 2002. Calcium at the crossroads of signaling. Plant Cell 14 Suppl, S401-17. https://doi.org/10.1105/tpc.002899

Sharma, A., Verma, R.K., 2019. Biofortification: A Promising Approach Toward Eradication of Hidden Hunger, in: Microbial Interventions in Agriculture and Environment. Springer Singapore, Singapore, pp. 313–327. https://doi.org/10.1007/978-981-13-8391-5_12

Sharma, D., Jamra, G., Singh, U.M., Sood, S., Kumar, A., 2017. Calcium biofortification: Three pronged molecular approaches for dissecting complex trait of calcium nutrition in finger millet (*Eleusine coracana*) for devising strategies of enrichment of food crops. Front. Plant Sci. 7, 2028. https://doi.org/10.3389/fpls.2016.02028

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012, 1–26. https://doi.org/10.1155/2012/217037

Shigaki, T., Barkla, B.J., Miranda-Vergara, M.C., Zhao, J., Pantoja, O., Hirschi, K.D., 2005. Identification of a crucial histidine involved in metal transport activity in the Arabidopsis cation/H^+ exchanger CAX1. J. Biol. Chem. 280, 30136–30142. https://doi.org/10.1074/jbc.M503610200

Srivastava, R.K., Pandey, P., Rajpoot, R., Rani, A., Gautam, A., Dubey, R.S., 2015. Exogenous application of calcium and silica alleviates cadmium toxicity by suppressing oxidative damage in rice seedlings. Protoplasma 252, 959–975. https://doi.org/10.1007/s00709-014-0731-z

Stephenson, P., Baker, D., Girin, T., Perez, A., Amoah, S., King, G.J., Østergaard, L., 2010. A rich TILLING resource for studying gene function in *Brassica rapa*. BMC Plant Biol. 10, 62. https://doi.org/10.1186/1471-2229-10-62

Stirbet, A., Lazár, D., Guo, Y., Govindjee, G., 2020. Photosynthesis: basics, history and modelling. Ann. Bot. 126, 511–537. https://doi.org/10.1093/aob/mcz171

Strasser, R.J., Tsimilli-Michael, M., Srivastava, A., 2004. Analysis of the Chlorophyll a Fluorescence Transient, in: Chlorophyll a Fluorescence. Springer Netherlands, Dordrecht, pp. 321–362. https://doi.org/10.1007/978-1-4020-3218-9_12

Sweetlove, L.J., Beard, K.F.M., Nunes-Nesi, A., Fernie, A.R., Ratcliffe, R.G., 2010. Not just a circle: flux modes in the plant TCA cycle. Trends Plant Sci. 15, 462–470. https://doi.org/10.1016/j.tplants.2010.05.006

Tan, W., Meng, Q. wei, Brestic, M., Olsovska, K., Yang, X., 2011. Photosynthesis is improved by exogenous calcium in heat-stressed tobacco plants. J. Plant Physiol. 168, 2063–2071. https://doi.org/10.1016/j.jplph.2011.06.009

Terashima, M., Petroutsos, D., Hüdig, M., Tolstygina, I., Trompelt, K., Gäbelein, P., Fufezan, C., Kudla, J., Weinl, S., Finazzi, G., Hippler, M., 2012. Calcium-dependent regulation of cyclic photosynthetic electron transfer by a CAS, ANR1, and PGRL1 complex. Proc. Natl. Acad. Sci. 109, 17717–17722. https://doi.org/10.1073/pnas.1207118109

Thakur, S., Choudhary, S., Bhardwaj, P., 2019. Comparative Transcriptome Profiling Under Cadmium Stress Reveals the Uptake and Tolerance Mechanism in Brassica juncea. J. Plant Growth Regul. 38, 1141–1152. https://doi.org/10.1007/s00344-019-09919-8

Tian, S., Lu, L., Labavitch, J., Yang, X., He, Z., Hu, H., Sarangi, R., Newville, M., Commisso, J., Brown, P., 2011. Cellular sequestration of cadmium in the hyperaccumulator plant species *Sedum alfredii*. Plant Physiol. 157, 1914–25. https://doi.org/10.1104/pp.111.183947

Tian, S., Xie, R., Wang, H., Hu, Y., Ge, J., Liao, X., Gao, X., Brown, P., Lin, X., Lu, L., 2016. Calcium Deficiency Triggers Phloem Remobilization of Cadmium in a Hyperaccumulating Species. Plant Physiol. 172, 2300–2313. https://doi.org/10.1104/pp.16.01348

Till, B.J., Datta, S., Jankowicz-Cieslak, J., 2018. TILLING: The Next Generation. Springer, Cham, pp. 139–160. https://doi.org/10.1007/10_2017_54

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N.E., Henikoff, J.G., Comai, L., Henikoff, S., 2003. Large-scale discovery of induced point mutations with high-

throughput TILLING. Genome Res. 13, 524–530. https://doi.org/10.1101/gr.977903

Tran, T.A., Popova, L.P., 2014. Functions and toxicity of cadmium in plants: recent advances and future prospects. Turk. J. Botany 37, 1–13. https://doi.org/10.3906/bot-1112-16

Wakeel, A., Farooq, M., Bashir, K., Ozturk, L., 2018. Micronutrient Malnutrition and Biofortification: Recent Advances and Future Perspectives, in: Plant Micronutrient Use Efficiency. Academic Press, pp. 225–243. https://doi.org/10.1016/b978-0-12-812104-7.00017-4

Wan, H., Chen, L., Guo, J., Li, Q., Wen, J., Yi, B., Ma, C., Tu, J., Fu, T., Shen, J., 2017. Genome-wide association study reveals the genetic architecture underlying salt tolerance-related traits in rapeseed (*Brassica napus* L.). Front. Plant Sci. 8, 593. https://doi.org/10.3389/fpls.2017.00593

White, P.J., Broadley, M.R., 2009. Biofortification of crops with seven mineral elements often lacking in human diets - iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol. 182, 49–84. https://doi.org/10.1111/j.1469-8137.2008.02738.x

White, P.J., Broadley, M.R., 2003. Calcium in plants. Ann. Bot. 92, 487–511. https://doi.org/10.1093/aob/mcg164

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Wu, Q., Shigaki, T., Williams, K.A., Han, J.S., Kim, C.K., Hirschi, K.D., Park, S., 2011. Expression of an Arabidopsis Ca^{2+}/H^{+} antiporter CAX1 variant in petunia enhances cadmium tolerance and accumulation. J. Plant Physiol. 168, 167–173. https://doi.org/10.1016/j.jplph.2010.06.005

Xiao, F., Yang, Z.Q., Lee, K.W., 2016. Photosynthetic and physiological responses to high temperature in grapevine (*Vitis vinifera* L.) leaves during the seedling stage. J. Hortic. Sci. Biotechnol. 92, 2–10. https://doi.org/10.1080/14620316.2016.1211493

Xu, W., Huang, W., 2017. Calcium-dependent protein kinases in phytohormone signaling pathways. Int. J. Mol. Sci. 18. https://doi.org/10.3390/ijms18112436

Yang, H., Jie, Y., Shao, X., 2011. Regulation of calcium uptake and translocation in plants. Symp. Integr. Canopy, Rootstock Environ. Physiol. Orchard Syst. 1011–1016

Yang, L., Wang, X., Chang, N., Nan, W., Wang, S., Ruan, M., Sun, L., Li, S., Bi, Y., 2019. Cytosolic Glucose-6-Phosphate Dehydrogenase Is Involved in Seed Germination and Root Growth Under Salinity in *Arabidopsis*. Front. Plant Sci. 10, 182. https://doi.org/10.3389/fpls.2019.00182

Zhang, Y., Zhang, G., Liu, L., Zhao, K., Wu, L., Hu, C., Di, H., 2011. The role of calcium in regulating alginate-derived oligosaccharides in nitrogen metabolism of *Brassica campestris* L. var. utilis Tsen et Lee. Plant Growth Regul. 64, 193–202. https://doi.org/10.1007/s10725-010-9557-7

Zhang, Y.H., Yin, H., Liu, H., Wang, W.X., Wu, L.S., Zhao, X.M., Du, Y.G., 2013. Alginate oligosaccharides regulate nitrogen metabolism via calcium in *Brassica campestris* L. var. *utilis* Tsen et Lee. J. Hortic. Sci. Biotechnol. 88, 502–508. https://doi.org/10.1080/14620316.2013.11512998

Zhang, Z.S., Peng, X.X., 2016. Multifunctional roles of photorespiration and its regulation for the balance. https://doi.org/10.13592/j.cnki.ppj.2016.1017

Zhao, J., Barkla, B.J., Marshall, J., Pittman, J.K., Hirschi, K.D., 2008. The Arabidopsis cax3 mutants display altered salt tolerance, pH sensitivity and reduced plasma membrane H^+-ATPase activity. Planta 227, 659–669. https://doi.org/10.1007/s00425-007-0648-2

Zorrilla, C., Schabow, J.E., Chernov, V., Palta, J.P., 2019. CAX1 vacuolar antiporter overexpression in potato results in calcium deficiency in leaves and tubers by sequestering calcium as calcium oxalate. Crop Sci. 59, 176–189. https://doi.org/10.2135/cropsci2018.06.0355

Zorrig, W., Rouached, A., Shahzad, Z., Abdelly, C., Davidian, J.-C., Berthomieu, P., 2010. Identification of three relationships linking cadmium accumulation to cadmium tolerance and zinc and citrate accumulation in lettuce. J. Plant Physiol. 167, 1239–1247. https://doi.org/10.1016/j.jplph.2010.04.012