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

**Abstract**

Cation/H+ exchanger transporters (CAXs) are crucial in Ca homeostasis and in the generation of Ca profiles involved in signalling processes. Given the crucial role of CAX1 in Ca 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 doses on plant metabolism. For this, the mutants and the parental line were grown under low, control and high Ca 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 to metabolism especially under high Ca 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 dose. This tolerance could be provided by an improved N and TCA metabolisms enzymes, and by a higher glutamate, malate, indole-3-acetic acid and abscisic acid concentrations. Therefore, *BraA.cax1a-12* mutation could be useful in *B. rapa* improving and the metabolomics changes observed in this mutant could be key for a greater tolerance to high Ca doses.

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

**Abbreviations**:AAs, amino acids; CAXs, cation/H+ exchangers transporters; PCA, principal components analysis; TCA tricarboxylic acid.

**1. Introduction**

One of the main mechanisms that plants possess to regulate internal Ca homeostasis is through plant cation/H+ exchangers (CAXs). CAXs transporters are crucial to generate Ca profiles involved in signalling processes mediated by this cation (Pittman and Hirschi 2016). Thus, Ca 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. Within the potential targets two very important are nitrogen (N) metabolism and tricarboxylic acid (TCA) enzymes (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 (Kusano et al. 2011). Several researches showed that Ca availability and homeostasis can affect N metabolism. Thus, deficient or toxic Ca applications cause the inhibition of N metabolism enzymes (Gao et al. 2011; Chao et al. 2008). Not only is important the Ca concentration but its homeostasis. Thus, the application of compounds such alginate oligosaccharides enhance N metabolism and total N concentration by improving Ca flux to the cytosol (Zhang et al. 2013; Xu et al. 2014). 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 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 CO2 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 besides being intermediaries in the TCA cycle they are 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 fluxes. Actually, Ca takes part in several important processes and regulatory functions in mitochondria including the regulation of some TCA dehydrogenases. In turn, Ca also indirectly determines its activity by joining substrates of TCA enzymes (Araújo et al. 2012).

Besides Ca, phytohormones are another of the fundamental elements that act together with it in the regulation of plant metabolism.Phytohormones are compounds with diverse structure that regulate plant metabolism in general, highlighting the effect of cytokinins (CKs) on N metabolism (Sakakibara et al. 2006). Likewise, phytohormones 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 affect to phytohormone synthesis and action (Marschner, 2012). On the other hand, Ca is able to activate hormone synthesis and acts in signalling cascades in response to phytorhomones. In this manner, Ca is involved in abscisic acid (ABA), gibberellins (GAs), indole-3-acetic acid IAA and ethylene signalling through protein kinases proteins that present a Ca binding domain (Xu and Huang, 2017).

Given the crucial role of Ca homeostasis in the regulation of plant metabolism and the role of CAX1 in Ca homeostasis, CAX1 modifications could produce effects on plant metabolism. CAX1 activity is regulated by fosforilation or protein binding to its N-terminal autoinhibitory domain. Some experiments achieved an enhancement of CAX1 activity through the substitution of certain AAs by other in its structure (Pittman et al. 2002; Mei et al. 2007; Zhao et al. 2009). 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*: *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. 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 KNO3, 3 mM NH4NO3, 2 mM MgSO4 • 7 H2O, 6 mM KH2PO4, 1 mM NaH2PO4 • 2 H2O, 2 μM MnCl2 • 4 H2O, 0.25 μM CuSO4 • 5 H2O, 0.1 μM Na2MoO4 • 2 H2O, 5 µM Fe-chelate (Sequestrene; 138FeG100), and 10 µM H3BO3. 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 CaCl2 as low Ca dose, 4 mM of CaCl2 as control Ca dose, and 40 mM of CaCl2 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*

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 and N forms*

For the Ca concentration determination, a sample of 150 mg dry material was subjected to a mineralization process with sulfuric acid and H2O2 by the method of Wolf (1982) and then Ca concentration was determined by ICP- MS.

NO3- 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 NO3- determination and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO3- concentration by spectrophotometry (Cataldo et al. 1975). NH4+ 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 KH2PO4 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 MgCl2, 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 †C18 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 NaHCO3, 5 mM MgCl2, 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 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 employed to assess relationships between treatments and all parameters analysed. All statistical analyses were carried out employing the Statgraphics Centurion XVI software.

**3. Results**

*3.1. Biomass and Ca concentration*

Plants grown under low and control Ca doses did not present significant differences in leaf DW between mutants and R-o-18. High Ca dose caused a biomass reduction in all lines in comparison to control Ca dose. However, *BraA.cax1a-12* plants retained more shoot DW when they received the highest Ca concentration (Fig. 1A).

Concerning foliar Ca accumulation, when low Ca was applied no significant differences were observed between lines. Nevertheless, as Ca supply increased, the differences between lines in their foliar Ca concentrations were more remarkable. Likewise, under control Ca dose mutants accumulated more foliar Ca than R-o-18 plants, being *BraA.cax1a-7* the mutant that presented the higher Ca concentration. Furthermore, when plants received the highest Ca dose, *BraA.cax1a-4* and *BraA.cax1a-7* mutants presented the higher Ca levels, followed by *BraA.cax1a-12*, and R-o-18 that registered the lower Ca concentration (Fig. 1B).

*3.2. N metabolism and photorespiration*

Both low and high Ca applications reduced NR in all lines in comparison with control Ca dose (Table 1) while GS activity only was reduced by high Ca dose (Table 2). Comparing between lines, under low Ca dose, we observed no significant differences in NR and AAT activities and in total reduced N. In addition, NO3- levels were higher in *BraA.cax1a-12* plants, NH4+ concentration increase in *BraA.cax1a-7* and *BraA.cax1a-12* plants while in these two mutants GDH activity decreased. Likewise, GOGAT activity decreased in *BraA.cax1a-4* and *BraA.cax1a-7* plants while GS activity increased in *BraA.cax1a-4* in comparison to R-o-18 plants. (Table 1 and 2). Under control Ca dose, plants did not present differences in GDH, GS and GOGAT activities. *BraA.cax1a-4* and *BraA.cax1a-7* presented lower NO3-, NR and higher NH4+ and AAT levels, while total reduced N was lower in *BraA.cax1a-4* and higher in *BraA.cax1a-7* and *BraA.cax1a-12* in comparison to R-o-18 plants (Table 1 and 2). High Ca dose caused a decrease in NO3- and total reduced N concentrations in *BraA.cax1a-4* and *BraA.cax1a-7* mutants. In *BraA.cax1a-7* we measured a lower NR and GOGAT activities and higher NH4+ concentration and GDH and AAT activities. Finally, *BraA.cax1a-12* plants presented greater NO3-, NR, total reduced N, GS, GOGAT and AAT levels and lower NH4+ concentration in comparison to R-o-18 plants (Table 1 and 2).

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

*3.3. Amino acid profile*

When mutant plants were subjected to low Ca dose, total AAs concentration increased especially in *BraA.cax1a-12* plants in comparison to R-o-18 plants. Proline (Pro) concentration decreased in *BraA.cax1a-4* and *BraA.cax1a-7* plants. Under control Ca dose, no differences in total AAs concentration were observed between lines. However, highlight that all mutants registered higher glycine (Gly), threonine (Thr) and Pro levels and lower of valine (Val) and isoleucine (Ile). Under high Ca dose, total AAs levels decreased in *BraA.cax1a-12* with the exception of Glu and histidine (His) with no changes. In the rest of mutants highlight the increase in Gly levels and the decrease in Val and Ile concentrations in comparison to R-o-18 plants (Table 3).

*3.4. OAs concentration and TCA cycle enzyme activities*

*BraA.cax1a* mutations affected to OAs concentrations in a Ca dose dependent way. Low Ca application unequally affected to citrate concentration depending on the *BraA.cax1a* mutation while all mutations increased malate (Fig. 3A and 3B). Oxalate levels were higher in *BraA.cax1a-7* and *BraA.cax1a-12* plants (Fig. 3C). Under control Ca conditions *BraA.cax1a-4* and *BraA.cax1a-7* decreased citrate and oxalate concentrations and *BraA.cax1a-12* did not affect to citrate and malate concentrations while decreased oxalic acid concentration with respect to R-o-18 plants. On the other hand, high Ca dose caused a reduction of OAs in all genotypes in comparison to control Ca dose, except in *BraA.cax1a-12* that maintained oxalate and increased malate concentrations under this dose (Fig. 3). Finally, *BraA.cax1a-4* and *BraA.cax1a-7* presented very low concentration of oxalate under high Ca dose (Fig. 3C).

Regarding TCA enzyme activities, CS activity was not especially affected by *BraA.cax1a* mutations, only was reduced by *BraA.cax1a-7* under low Ca dose and increased by *BraA.cax1a-12* mutation under high Ca dose. Conversely, all mutations increased MDH activity under high Ca dose. Regarding PEPC activity highlight its increase in *BraA.cax1a-12* plants under both low and high Ca conditions (Table 4).

*3.5. Phytohormone profile*

In comparison to R-o-18 plants, *BraA.cax1a*mutants presented higher IAA levelsunder all Ca doses. Regardless the Ca dose, *BraA.cax1a-4* plants presented no significant differences in ABA and ACC levels in comparison to R-o-18 plants. Under low Ca dose, phytohormones increased their levels in *BraA.cax1a-7* and *BraA.cax1a-12* plants. Furthermore, under control dose in mutants only IAA increased while the rest of hormones registered similar values to R-o-18 or decreased. On the other hand, under high Ca dose highlight that in *BraA.cax1a-12* increased all phytohormones except for GAs that did not change and a decrease in ACC concentration. Finally, in *BraA.cax1a-4* and *BraA.cax1a-7* we observed an increment in CKs levels and a decrease in GAs while ACC decreased in *BraA.cax1a-7* plants (Table 5).

*3.6. Principal components analysis*

Principal components analysis (PCA) was employed 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 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.4).

The PCA score scatter plot show that plants subjected to high Ca dose are clearly separated from control and low doses. When plants were grown under low Ca, *BraA.cax1a-12* separated from the other lines that did not show great differences between themselves. Under control Ca 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 dose lines were well separated (Fig. 4A).

The plot of component weights showed strong relationships between foliar Ca, Pro, ACC, Ser, CKs, tZ, total AAs, Thr and ABA. The parameters related to foliar DW were NR, GS, GDH, citrate, oxalate, malate, PEPC and AIA. As expected NO3-, N total and Glu were close related. On the other hand GOGAT were related to the concentration of some AAs as Val, Ala and Ile. Finally, MDH, GO, GGAT and AAT also were close related (Fig. 4B).

**4. Discussion**

*4.1. Biomass and Ca concentration*

Several authors evidenced that modifications in CAX1 activity affect to Ca homeostasis and produce effects in plant physiology. Thus, *A. thaliana* *cax1* knockout mutants showed an altered growth and a decrease in Ca concentration especially when were grown under high Ca dose (Catalá et al. 2003; Conn et al. 2011). On the other hand, plants with higher CAX1 activity accumulated more Ca and present or not an altered phenotype, depending on the CAX1 modification and the species (Hirschi 1999; Park et al. 2005; Morris et al. 2008).The mutants used in the present experiment did not present great alterations in their growth (Fig. 1). Indeed, when high Ca dose was applied, it is noteworthy that *BraA.cax1a-12* showed a lower foliar biomass loss and registered 82% more biomass than R-o-18 plants. In the rest of lines this Ca dose caused a great reduction in foliar biomass in comparison to control Ca dose (Fig. 1A). These results indicate that *BraA.cax1a-12* mutants could present some mechanism to avoid the Ca toxicity observed in the rest of lines. Likewise, *BraA.cax1a* mutations improved Ca accumulation especially under high Ca dose supply. However, this accumulation was lower in *BraA.cax1a-12*plants (Fig. 1B) that could store more efficiently Ca in vacuoles as it was observed in other experiment in which a higher CAX1 activity enhance Ca storage in this organelle (Pittman and Hirschi, 2016). This could provide *BraA.cax1a-12* a better tolerance to Ca toxicity.

*4.2. N metabolism and photorespiration*

It is well known that a proper Ca supply and Ca internal homeostasis is basic for an optimal N metabolism performance (Gao et al. 2011; Chao et al. 2008; Zhang et al. 2013; Xu et al. 2014). Thus, *BraA.cax1a* mutations could altered N metabolism because CAX1 is essential for Ca homeostasis. In addition this is supported by Wang et al. (2000) that proved that *CAX1* expression is related to NO3- concentration and an increase in its concentration could enhance *CAX1* expression. In general, the present study results agree with these ideas because the low and higher Ca doses caused alterations in N metabolism. Thus, high Ca dose was the dose that most reduced NO3- concentration and NR activity in all lines (Table 1). Regarding GDH, GS, and GOGAT enzymes, in general, we only observe differences between R-o-18 and mutants under low and high Ca doses (Table 2). This suggest that *BraA.cax1a m*utations only affect to NH4+ assimilation process when non-optimal levels of Ca are applied to plants.

Considering *BraA.cax1a* mutations, *BraA.cax1a-7* clearly produced an alteration in N metabolism. This mutation reduced NO3- accumulation and GS/GOGAT cycle enzyme activities and increased NH4+ concentration and photorespiration enzymes (Tables 1 and 2; Fig. 2). These results are indicative of a high stress in this mutant probably caused by an alteration in Ca fluxes. The lack of NADH and ATP produced by an altered photosynthesis performance (Navarro-León et al. 2018) might be a possible reason for the lower enzyme activities in *BraA.cax1a-7* plants under control and high Ca conditions. The inhibition of N metabolism enzymes could be the cause of the lower total reduced N concentration in *BraA.cax1a-7* under higher Ca dose (Table 1 and 2). In contrast, *BraA.cax1a-12* plants did not presented an altered N metabolism under low and control Ca doses and enhanced it under high Ca dose (Table 1 and 2). Kim et al. (2011) observed a direct correlation between NO3- and foliar N concentrations and foliar DW, so NO3- bioavailability is able to limit plant biomass. In *BraA.cax1a-12* this fact could be important since it present higher NO3- levels. In addition, under higher Ca dose *BraA.cax1a-12* was able to maintain a higher NR in comparison to the other lines (Table 1). Therefore, a higher NO3-concentration could be enhancing NR activity, this would lead to a higher N assimilation rate and thereby could explain the greater both total reduced N (Table 1) and DW we observed in these plants (Fig. 1A).

According our results, *BraA.cax1a-7* mutant could have an enhanced GDH activity for a fast NH4+ assimilation preventing its toxicity under high Ca 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 conditions since a higher GDH activity in *BraA.cax1a-4* and *BraA.cax1a-7* mutants (Table 2) could reassimilate the excess of NH4+ produced by photorespiration. Conversely, the lower GDH in *BraA.cax1a-7* and *BraA.cax1a-12* under low Ca supply (Table 2) could be the reason why NH4+ is accumulated in theseplants (Table 1). There results support the role of Ca in GDH activity to detoxify NH4+ excess. On the other hand, it was observed that photorespiration can be an important source of NH4+ and even the amount of NH4+ produced can overcome NH4+ produced by NR activity (Wingler et al. 2000). Therefore, the enhacenment in phtorespiration could cause the increment in NH4+ concentration. However, under low and high Ca concentrations in spite GO and GGAT also increase in *BraA.cax1a-4* (Fig. 2) we did not observe an increase in NH4+ concentration (Table 1) and this could be due the higher GS activity presented in this mutant (Table 2). Likewise, photorespiration might be activated by the higher reactive oxygen species (ROS) concentration that we observed in *BraA.cax1a-4* and *BraA.cax1a-7* mutants (Navarro-León et al. 2018). A higher photorespiration rate usually is an adaptive response of plants suffering some kind of stress (Zhang and Peng, 2016). There are not enough studies about the influence of Ca supply on photorespiratory enzymes in plants. However, Ca fluxes in peroxisomes are crucial in Ca homeostasis and this is important to activate enzymes such as catalase (Costa et al. 2013). Recently, Corpas and Barroso (2017) demonstrated that Ca and calmoduline are necessary to GO activation in peroxisomes. Therefore, the different *BraA.cax1a* mutation might affect these Ca fluxes differently and consequently the activity of photorespiratory enzymes.

*4.3. Amino acid profile*

PCA analysis related Ca concentration to total AAs, and specifically Pro, Ser and Thr (Fig. 4B) because plants with high Ca levels presented high levels of these AAs (Table 3). A decrease in growth as we observed under high Ca dose usually causes an accumulation of AAs in the cytoplasm due to a decrease in the protein synthesis/degradation ratio (Atilio and Causin, 1996). 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 deficiency reduced free AAs. This reduction also occurs in R-o-18 plants that presented lower AAs concentration under low Ca but not in mutant lines (Table 3), so mutations could maintain AAs levels under Ca deprivation. In contrast with the majority of AAs, *BraA.cax1a* mutations reduced Val and Ile levels under control and high Ca doses (Table 3). This reduction could be related to the fact that the synthesis and the catabolism of these AAs is close related. For intstancebranched-chain aminotransferases degradate these AAs in the mitochondria and could be activated as a consquence of *BraA.cax1a* mutations (Schuster and Binder, 2005).

PCA analysis also related NH4+ concentration with Arg and His levels (Fig. 4B). For these three parameters we observed a great increase in *BraA.cax1a-7* under low Ca doses and a decrease in *BraA.cax1a-12* under high Ca dose (Table 3). Arg and His are the 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 (Galiliet al. 2016). In this manner, the increase in Asp levels in *BraA.cax1a-4* and *BraA.cax1a-*7 plants (Table 3) is also supported by a higher AAT activity (Table 2). Therefore, is probably that in *BraA.cax1a-7* could exist 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.

Likewise, *BraA.cax1a-12* registered higher Glu than the other lines (Table 3). 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, NO3- and total reduced N (Fig. 4B) and this could be because Glu is one of the most concentrated AAs and its synthesis is directly related with GS/GOGAT enzymes that are activated by NO3- levels. Thus, *BraA.cax1a-12* mutant presented the greatest levels for these parameters (Table 1 and 2). 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; Obata and Fernie 2012). All plants in our experiment showed a clear increase in Pro and Thr accumulations caused by Ca toxicity. However this increase was much lower in *BraA.cax1a*-*12* plants which indicates lower stress in this mutant (Table 3).

*4.4. OA concentrations 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 dose in comparison to control Ca dose, probably in order to increase Ca uptake. However, under high Ca dose, OAs did not appear to improve Ca homeostasis since their concentrations decreased markedly, except for malate in *BraA.cax1a-12* mutant (Fig. 3). Malate could be key in the tolerance of high Ca levels because of is the main OA that binds to Ca in vacuoles. In addition malate is key in NADH transport through malate antiporters and to it has a positive influence on N metabolism (Igamberdiev and Eprintsev, 2016). This could be important in *BraA.cax1a-12* mutant to cope with Ca toxicity. Likewise, this mutant maintained citrate levels in contrast with the other mutants in which citrate levels decreased under control and high Ca doses (Fig. 3B). On the other hand, oxalate crystal formation has a function of Ca storage in vacuoles, as well as the maintenance of concentration gradient between the vacuole and the cytosol (Igamberdiev and Eprintsev, 2016). In the present experiment oxalate levels decreased in plant grown under the high Ca dose (Fig. 3C). This could be positive preventing the generation of Ca-oxalate crystals that could be toxic for humans or animals (Dayod et al. 2010).

Regarding TCA cycle, Ca supply has proved to affect to TCA cycle enzymes in *B. rapa* plant (Blasco et al. 2015) and other species (Su et al. 2016; He et al. 2015). *BraA.cax1a* mutations affected unevely to TCA enzymes. However, *BraA.cax1a-12* enhaced CS and PEPC activites under Ca toxicity (Table 4), which could contribute to maintain a higher biomass in this mutant. An increase in TCA activity may be good against stress due to Ca toxicity. More specifically, PEPC activity is involved in several physiological processes and acts as linking factor between C and N metabolisms (Sweetlove et al. 2010). The greater PEPC activity in *BraA.cax1a-12* in both conditions of deficiency and especially of Ca toxicity (Table 4) can be a positive response of tolerance to stress. This connects with N metabolism as it produces OAA that is used by AAT enzyme that increases in *BraA.cax1a-12* as well (Table 2). 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 3) to be catabolized in TCA cycle to produce energy. These mutant would have energy shortage due to the lower photosyntesis perfomance as observed by Navarro-León et al. (2018) and could compesate this with the catabolism of AAs in TCA.

*4.5. Phytohormone profile*

Being that Ca is involved in phytohormone signalling and synthesis (Xu and Huang, 2017), CAX1mutations could affect both concentration and responses of phytorhomones in plants (Xia et al. 2018).In factCheng et al. (2003)observed that IAA signalling is affected 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 dose applied (Table 5). IAA synthesis and accumulation could be close related to Ca fluxes produced by CAX1 transporters. On the other hand, *BraA.cax1a-7* and *BraA.cax1a-12* increased phytohormone levels under low Ca dose (Table 5). These two mutants presented high average DW (Fig. 1A), although not significant, this mutation could enhance tolerance to Ca deficiency.

According PCA analysis IAA levels are related to foliar DW (Fig. 4B). The lower IAA levels are found under high Ca dose as well as foliar DW. In addition, *BraA.cax1a-12* presented the highest IAA levels and also the highest biomass (Table 5; Fig. 1A). Thus, this suggest that IAA could be key to maintain biomass under high Ca conditions. On the other hand, under high Ca dose CKs levels correlated well with Ca accumulation, mutants accumulated more Ca and presented higher CKs level. *BraA.cax1a-12* presented less CKs and less Ca than the other mutant lines (Table 5; Fig. 1B), suggesting that high CKs levels could stimulate Ca 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 metabolism (Sakakibara et al. 2006). In our study the stress produced by Ca might induce CKs synthesis or accumulation in order to induce these genes. In similar way to CKs,GAs and specifically GA3 affect to Ca accumulation in some species (Yang et al. 2011). However, in the present experiment GAs concentration was not related with Ca accumulation (Fig. 4B). *BraA.cax1a-12* was the unique line that maintained its GAs levels while in the rest of line GAs levels decreased under high Ca application with respect its control levels.

Regarding stress related hormones, such as ABA and ethylene,*BraA.cax1a-4* mutation, regardless the Ca dose, presented the same levels of these hormones than R-o-18 in contrast to the other mutants (Table 5). Therefore, not all *BraA.cax1a* mutations affected to phytohormone levels. ABA induces cytosolic Ca2+ elevation, which is in part due to Ca2+ release from the vacuole where CAX1 transporter is found. That is why ABA could affect to cellular Ca distribution and to Ca concentration in plants (De Freitas et al. 2013).Likewise, ABA is a stress related hormone and is known to reduce plant growth. However, ABA could help to cope with stress in *BraA.cax1a-12* mutant or in this mutant high ABA levels could be compensated by the great increase in IAA (Table 5). On the other hand, ethylene, gaseous hormone synthetized from ACC, increase its synthesis when plants are under stress. In addition, 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 concentration may be related to ACC because this hormone increased with Ca concentration (Fig. 4B). However, it can not be stated that ACC promotes Ca accumulation because R-o-18 presented the higher ACC levels but the lower Ca accumulation when grew under high Ca dose (Table 5; Fig. 1B).

**5. Conclusions**

The results obtained in this work show that *BraA.cax1a* mutations clearly affect to plant metabolism. These effects depend on the mutation in CAX1a transporter and were more remarkable when plant grow under high Ca dose. Thus, *BraA.cax1a-4* and specially *BraA.cax1a-7* mutations probably produce alterations in Ca 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 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 biofortification programs employing *B. rapa* plants and the metabolomics changes observed in this mutant could be key for a greater tolerance to high Ca doses.

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**Tables**

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

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | NO3-  (mg g-1 DW) | NR  (µM NO2 mg prot-1 min-1) | NH4+  (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 |
|  | LSD0.05 | 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* | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 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 | \*\*\* | \*\*\* | \*\* | \*\*\* |
|  | LSD0.05 | 10.19 | 0.07 | 0.17 | 12.36 |
| Analysis of variance | | | | |  |
| Doses (D) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\*\* | \* | NS |
| D x M |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 |  | 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 (\*\*\*).

**Table 2.** Activities of N metabolism enzymes in *BraA.cax1a* mutants and R-o-18 plants grown under three Ca doses

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | GDH  (ΔAbs mg prot-1min-1) | GS  (ΔAbs mg prot-1min-1) | GOGAT  (ΔAbs mg prot-1min-1) | AAT  (ΔAbs mg prot-1min-1) |
| 0.4 mM | R-o-18 | 0.37a | 96.36b | 0.40a | 0.18a |
|  | *BraA.cax1a-4* | 0.35ab | 118.77a | 0.28b | 0.17a |
|  | *BraA.cax1a-7* | 0.27c | 77.69c | 0.27b | 0.26a |
|  | *BraA.cax1a-12* | 0.30bc | 91.55bc | 0.38a | 0.24a |
|  | *p*-value | \*\* | \*\*\* | \*\* | NS |
|  | LSD0.05 | 0.05 | 16.92 | 0.09 | 0.09 |
| 4 mM | R-o-18 | 0.26a | 99.41ab | 0.36a | 0.23b |
|  | *BraA.cax1a-4* | 0.29a | 99.89ab | 0.31a | 0.29a |
|  | *BraA.cax1a-7* | 0.26a | 107.33a | 0.37a | 0.33a |
|  | *BraA.cax1a-12* | 0.27a | 88.79b | 0.34a | 0.20b |
|  | *p*-value | NS | NS | NS | \*\*\* |
|  | LSD0.05 | 0.04 | 15.73 | 0.11 | 0.05 |
| 40 mM | R-o-18 | 0.16bc | 17.83c | 0.50b | 0.26c |
|  | *BraA.cax1a-4* | 0.21ab | 28.57b | 0.49b | 0.31bc |
|  | *BraA.cax1a-7* | 0.26a | 20.08c | 0.29c | 0.52ª |
|  | *BraA.cax1a-12* | 0.15c | 60.85a | 0.66a | 0.45ab |
|  | *p*-value | \*\*\* | \*\*\* | \*\*\* | \*\* |
|  | LSD0.05 | 0.05 | 8.39 | 0.12 | 0.15 |
| Analysis of variance | | | | | |
| Doses (D) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | \* | \* | \*\*\* | \*\*\* |
| D x M |  | \*\*\* | \*\*\* | \*\*\* | \*\* |
| LSD0.05 |  | 0.03 | 8.65 | 0.06 | 0.06 |

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 (\*\*\*).

**Table 3.** Amino acids concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Total AAs | Pro | Thr | Ser | Val | Ile | His | Arg | Gly | Asp | Glu |
| 0.4 mM | R-o-18 | 33.23c | 2.32b | 2.77c | 5.54b | 0.53c | 0.09c | 0.89c | 9.06c | 0.59d | 3.77bc | 7.67b |
|  | *BraA.cax1a-4* | 55.01b | 1.39c | 5.78ab | 12.21a | 0.73b | 0.22b | 2.34bc | 14.63b | 3.76b | 5.07a | 8.91b |
|  | *BraA.cax1a-7* | 75.88ª | 1.67c | 4.57b | 8.53ab | 0.93a | 0.31a | 7.76a | 32.18a | 5.59ª | 4.71ab | 9.63ab |
|  | *BraA.cax1a-12* | 59.55b | 4.00a | 6.17a | 12.09a | 0.82ab | 0.13c | 2.99b | 14.44b | 3.39c | 3.51c | 11.99a |
|  | p-value | \*\*\* | \*\*\* | \*\* | \* | \* | \*\* | \*\* | \*\*\* | \*\* | \* | \* |
|  | LSD0.05 | 7.60 | 0.45 | 1.41 | 3.84 | 0.17 | 0.07 | 1.68 | 1.71 | 0.31 | 1.11 | 2.53 |
| 4 mM | R-o-18 | 51.42ª | 0.48d | 4.47c | 10.87a | 1.01a | 0.51a | 1.43c | 12.39a | 0.78d | 3.82b | 8.82bc |
|  | *BraA.cax1a-4* | 57.17ª | 6.88a | 6.82ª | 12.37a | 0.50c | 0.16b | 1.59bc | 10.16a | 4.31b | 4.69ab | 9.72b |
|  | *BraA.cax1a-7* | 49.37ª | 1.12c | 5.11b | 12.57a | 0.59c | 0.19b | 2.14b | 10.84a | 6.43a | 4.95a | 7.60c |
|  | *BraA.cax1a-12* | 54.77ª | 3.66b | 6.68a | 11.95a | 0.79b | 0.12b | 2.92a | 12.70a | 3.54c | 3.82b | 11.90a |
|  | p-value | NS | \*\* | \* | NS | \* | \*\*\* | \*\* | NS | \*\*\* | \* | \*\* |
|  | LSD0.05 | 17.07 | 0.62 | 0.60 | 4.14 | 0.17 | 0.09 | 0.63 | 0.76 | 0.56 | 0.88 | 1.70 |
| 40 mM | R-o-18 | 96.92ª | 34.06a | 9.01a | 20.84ab | 1.54a | 0.42a | 2.08a | 14.83a | 1.51c | 3.51b | 9.17b |
|  | *BraA.cax1a-4* | 95.92ab | 32.13a | 10.12a | 22.53a | 0.90bc | 0.30b | 1.99a | 12.16ab | 2.11b | 3.74b | 9.94ab |
|  | *BraA.cax1a-7* | 81.17b | 27.09ab | 7.19b | 18.16bc | 0.76c | 0.19c | 1.71ab | 10.66b | 3.63a | 4.30a | 5.60c |
|  | *BraA.cax1a-12* | 70.67c | 21.53b | 7.05b | 16.88c | 1.09b | 0.17c | 1.50b | 9.13b | 0.97d | 2.14c | 10.20a |
|  | p-value | \* | \* | \* | \* | \*\* | \* | \* | \* | \*\*\* | \*\* | \*\*\* |
|  | LSD0.05 | 10.27 | 7.43 | 1.78 | 3.19 | 0.31 | 0.10 | 0.40 | 3.52 | 0.45 | 0.52 | 0.89 |
| Analysis of variance | | | | |  |  |  |  |  |  |  |  |
| Doses (D) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \* |
| Mutation (M) |  | \* | \*\* | \*\*\* | \*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| D x M |  | \*\*\* | \*\*\* | \*\*\* | \*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | NS | \*\* |
| LSD0.05 |  | 6.66 | 2.01 | 0.63 | 1.69 | 0.10 | 0.04 | 0.48 | 2.02 | 0.21 | 0.43 | 0.83 |

Values are expressed as μmol g-1 FW 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. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

**Table 4** Activities of some TCA cycle enzymes in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | CS  (ΔAbs mg prot-1min-1) | MDH  (ΔAbs mg prot-1min-1) | PEPC  (ΔAbs mg prot-1min-1) |
| 0.4 mM | R-o-18 | 0.16ab | 0.76b | 0.19b |
|  | *BraA.cax1a-4* | 0.21a | 0.64c | 0.12c |
|  | *BraA.cax1a-7* | 0.10c | 0.82a | 0.14bc |
|  | *BraA.cax1a-12* | 0.16b | 0.38d | 0.26a |
|  | *p*-value | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 0.05 | 0.05 | 0.05 |
| 4 mM | R-o-18 | 0.11a | 0.79a | 0.17b |
|  | *BraA.cax1a-4* | 0.11a | 0.30b | 0.24a |
|  | *BraA.cax1a-7* | 0.12a | 0.82a | 0.09c |
|  | *BraA.cax1a-12* | 0.12a | 0.82a | 0.16b |
|  | *p*-value | NS | \*\*\* | \*\*\* |
|  | LSD0.05 | 0.05 | 0.11 | 0.03 |
| 40 mM | R-o-18 | 0.11b | 0.88b | 0.05b |
|  | *BraA.cax1a-4* | 0.10b | 1.02a | 0.02c |
|  | *BraA.cax1a-7* | 0.13ab | 1.03a | 0.02c |
|  | *BraA.cax1a-12* | 0.16a | 0.96a | 0.08a |
|  | *p*-value | \* | \*\* | \*\*\* |
|  | LSD0.05 | 0.04 | 0.07 | 0.02 |
| Analysis of variance | | | | |
| Doses (D) |  | NS | \*\*\* | NS |
| Mutation (M) |  | \*\* | \*\*\* | \*\*\* |
| D x M |  | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 |  | 0.02 | 0.05 | 0.02 |

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 (\*\*\*).

**Table 5.** Phytohormones concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | IAA | GA1 | GA3 | GA4 | GAs | tZ | iP | CKs | ACC | ABA |
| 0.4 mM | R-o-18 | 7.64d | 0.04b | nd | 0.02c | 0.06b | 171.28c | 2.28b | 173.56c | 163.71c | 5.89b |
|  | *BraA.cax1a-4* | 13.00c | 0.05ab | nd | 0.04b | 0.10b | 180.92bc | 1.54c | 182.46c | 171.58c | 4.81b |
|  | *BraA.cax1a-7* | 21.43b | 0.07a | 0.07 | 0.07ª | 0.21ª | 199.15b | 3.09a | 214.09b | 261.87b | 8.97a |
|  | *BraA.cax1a-12* | 33.29a | 0.08a | 0.16 | 0.02c | 0.25ª | 244.35a | 1.18c | 245.53ª | 389.48a | 8.24a |
|  | p-value | \*\*\* | NS |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\* | \*\*\* | \*\* |
|  | LSD0.05 | 3.95 | 0.03 |  | 0.01 | 0.05 | 21.16 | 0.66 | 23.54 | 70.01 | 2.04 |
| 4 mM | R-o-18 | 5.28c | 0.22a | nd | 0.03ab | 0.25ª | 216.56a | 2.07a | 218.62ª | 759.26a | 7.07a |
|  | *BraA.cax1a-4* | 8.91b | 0.10bc | nd | 0.05a | 0.15b | 244.201ª | 1.07b | 245.27ª | 744.03ª | 8.91a |
|  | *BraA.cax1a-7* | 10.58b | 0.17ab | 0.04 | 0.04ab | 0.25ª | 181.92b | 1.30b | 185.10b | 564.85b | 8.43a |
|  | *BraA.cax1a-12* | 16.63a | 0.07c | 0.05 | 0.03b | 0.14b | 215.47a | 2.55a | 218.03ª | 463.62c | 7.93a |
|  | p-value | \*\*\* | \*\* |  | NS | \* | \*\* | \*\*\* | \* | \*\*\* | NS |
|  | LSD0.05 | 3.62 | 0.08 |  | 0.02 | 0.09 | 29.07 | 0.56 | 29.82 | 84.34 | 1.90 |
| 40 mM | R-o-18 | 3.90d | 0.13a | nd | 0.02a | 0.15ª | 246.02d | 1.70c | 247.72d | 1298.51a | 14.59b |
|  | *BraA.cax1a-4* | 7.02b | 0.07bc | nd | 0.02a | 0.09b | 374.85a | 4.72b | 379.57ª | 1277.23a | 14.83b |
|  | *BraA.cax1a-7* | 5.31c | 0.03c | 0.03 | 0.02a | 0.08b | 335.25b | 2.84bc | 338.09b | 1082.08b | 9.90b |
|  | *BraA.cax1a-12* | 11.33a | 0.10ab | nd | 0.02a | 0.14ª | 291.08c | 3.55ab | 294.63c | 954.88c | 22.40a |
|  | p-value | \*\*\* | \* |  | NS | \*\* | \*\*\* | \* | \*\*\* | \*\* | \*\* |
|  | LSD0.05 | 1.40 | 0.05 |  | 0.01 | 0.04 | 29.02 | 1.64 | 29.45 | 70.37 | 5.89 |
| Analysis of variance | | | | |  |  |  |  |  |  |  |  |
| Doses (D) |  | \*\*\* | \*\*\* |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\* |  | \*\*\* | \*\*\* | \*\*\* | NS | \*\*\* | \*\*\* | \*\* |
| D x M |  | \*\*\* | \*\*\* |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 |  | 1.72 | 0.03 |  | 0.01 | 0.03 | 13.78 | 0.55 | 14.29 | 38.86 | 1.94 |

Values are expressed as ng 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. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

**Figures legends**

**Fig. 1.** Leaf biomass (A) and Ca concentration (B) in *BraA.cax1a* mutants and R-o-18 plants grown under 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).

**Fig. 2.** Effect of BraA.cax1a mutations and Ca doses on activity of GO (A) and GGAT (B) enzymes. 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).

**Fig. 3.** Effect of *BraA.cax1a* mutation and Ca 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).

**Fig. 4**. 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 doses (0.4, 4 and 40 mM)

**Fig. 1**





**Fig. 2.**





**Fig. 3**







**Fig. 4.**

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