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

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**ABSTRACT**

Crop production is facing the increasing salinity in soils and irrigation waters. This is a widespread problem that affects crops with high economic importance and severely reduces yields. Oxidative stress caused by salinity induces in plants complex defensive mechanisms involving the antioxidant system and osmoprotector compounds. In this strategy, Ca2+ plays a pivotal role in counteracting salt stress, and the modification of the Ca2+ 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. *BraA.cax1a* and parental line R-o-18 were grown under saline conditions and biomass, ions accumulation, antioxidant system, 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 Ca2+ and K+ in the shoot, an enhanced ROS scavenging (increased superoxide dismutase and catalase activities) and enhanced redox state, measured as ascorbate levels. In addition, 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 applied to enhance salt tolerance in crops.

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

**Abbreviations:** APX, ascorbate peroxidase; AS, absorption selectivity; ASA, ascorbate; GSH, glutathione; CAT, catalase; CAX, cation exchangers; DHA, dehydroascorbate; EC, electrolyte leakage; GPX, glutathione peroxidase; GR,glutathione reductase; GSSG, oxidized glutathione; HSP, heat shock proteins; LOX, lipoxygenase; MDA, malondialdehyde; SOD, superoxide dismutase; STI, salt tolerance index; TS, translocation selectivity

**1. INTRODUCTION**

Currently, salinity levels are increasing in cultured soils and irrigation waters, affecting crop production worldwide. This problem is present in about 20% of the irrigated land, 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 yield losses. In addition, the accumulation of Na+ ions leads to alterations in plant physiology, osmotic potential, nutritional imbalances and causes oxidative stress (Acosta-Motos et al., 2017).

One of the main symptoms of saline stress is the occurrence of oxidative stress with an accumulation of ROS (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 and biomass production (Adhikari et al., 2019). The disruption of the photosynthesis process produced under saline conditions leads to an abnormal energy flux through photosystems, in turn generating an excess of energy that usually produces ROS, causing photoinhibition. The toxic O⁠2˙‾ produced is subsequently converted in H2O2 by superoxide dismutase (SOD) (Munns and Gilliham, 2015). In addition, 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 are able to counteract the low ROS generation. However, under stress such as salinity, this equilibrium often is disturbed which leads to higher ROS accumulation (Acosta-Motos et al., 2017).

Two of the main mechanisms that plants use to cope with salinity are the distribution and compartmentalization of toxic ions (mainly 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 O⁠2˙‾, and catalase (CAT) and peroxidases that eliminate H2O2. In addition, enzymes of the ascorbate-glutathione (AsA/GSH) cycle such as glutathione reductase (GR) are able to regenerate non-enzymatic antioxidants (Acosta-Motos et al., 2017). Among the non-enzymatic compounds, ascorbate (AsA) and glutathione (GSH) play a main role because of their antioxidant properties (Sharma et al., 2012). GSH reduces 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 are able to 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 as a response to salt stress. Hence, Pro accumulation is an important indicator of salinity tolerance in plants (Gill and Tujeta, 2010).

Calcium (Ca2+) 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 Ca2+ in salinity tolerance has been extensively proved (Köster et al., 2019). Toxic Na+ concentrations hamper Ca2+ function due to the antagonism between these ions; besides, Ca2+ favors K+ uptake over Na+ (Parida and Das, 2005). Thus, Ca2+ homeostasis is crucial in salt tolerance: in response to stress, Ca2+ 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 Ca2+ fluxes in cells (Pokotilo 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(Pokotilo et al., 2012). CAX1 is the transporter located on vacuole membranes showing the highest Ca2+ transport activity. Three new variants of CAX1 were generated in *Brassica* *rapa* using TILLING (Targeting Induced Local Lesions in Genomes) in BraA.CAX1a transporter (Graham et al., 2014). 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) and photoperiod (14/10-h) and PAR (350 µmol m-2 s-1). The plants were watered with a growth solution composed of 6 mM KH2PO4, 4 mM KNO3, 4 mM Ca(NO3)2 • 4 H2O, 2 mM MgSO4 • 7 H2O, , 1 mM NaH2PO4 • 2 H2O, 10 µM H3BO3, 5 µM Fe-chelate (Sequestrene; 138FeG100), 2 μM MnCl2 • 4 H2O, 1 µM ZnSO4, 0.25 μM CuSO4 • 5 H2O, and 0.1 μM Na2MoO4 • 2 H2O. This solution had a pH of 5.5–6.0 and was renewed every three days.

*2.2. Experimental* *design and treatments*

Treatments were applied 30 days after germination and were maintained for 21 days. 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 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 Ca2+, Na+, and K+ concentrations and selectivity parameters*

Ca2+, 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 nitric acid (HNO3)/perchloric acid (HClO4) (v/v) and H2O2 at 30%. At the end of the mineralization process, 20 ml of mili-Q H2O were added and mineral elements 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(TSK, 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). The CAT activity was determined according to the method of Nakano and Asada (1981). The O2.− determination was performed according to the method of Kubiś (2008). H2O2 concentration was measured as described by Junglee et al. (2014). MDA concentration was determined according to Fu and Huang (2001).

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 (EC1) 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 (EC2) was measured. The percentage of electrolyte leakage was obtained using the following formula: (EC1/EC2) x 100.

*2.6. Lipoxygenase (LOX), peroxidases and GR activities*

LOX enzyme activity was recorded according to Minguez-Mosquera et al. (1993). Ascorbate peroxidase (APX) and GR activities were measured according to Rao et al. (1996). Glutathione peroxidase (GPX) activity was registered as described by Elia et al. (2003) with modifications using H2O2 as a substrate. 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). 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). 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. To the resulting supernatant was 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.

*2.10. 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*

Leaf DW did not change in all lines examined 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 percentage. Likewise, *BraA.cax1a-4* also presented higher STI than the rest of the plants. Under control conditions, root DW of *BraA.cax1a*-*7* was lower, whereas no significant differences were observed when lines were grown under salinity. However, *BraA.cax1a-4* and *BraA.cax1a-12* plants registered a higher percentage of reduction with respect 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 | \*\* |  |
|  | LSD0.05 | 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 | \* |
|  | LSD0.05 | 0.14 | 0.05 | 3.01 |
| Analysis of variance | | | | |
| Salinity (S) |  | \*\*\* | \*\*\* |  |
| Mutation (M) |  | \*\* | \*\* |  |
| S x M |  | NS | NS |  |
| LSD0.05 |  | 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 in relation 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 Ca2+ 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 Ca2+ 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 Ca2+ 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 led to a lower Na+/K+ 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* with the higher content (Fig. 1F). Regarding selectivity indexes, *BraA.cax1a-4* presented the lowest percentage of increment of ASK, Na (400% compared to more than 500% in the rest of lines) and the highest of ATK, Na (65% compared to -34% in R-o-18 plants). Furthermore, under salinity, *BraA.cax1a-7* and *BraA.cax1a-12* showed lower ASK, Na and *BraA.cax1a-12* registered a light increment in ATK, Na (14%) compared with control conditions (Fig. 1G-H).



**Figure 1**. Effect of R-o-18 and *BraA.cax1a* mutations on leaf Ca2+ (A), leaf Na+ (B), leaf K+ (C), Na+/K+ ratio (D), root Na+ (E), root K+ (F), ASK / Na (G), TSK / 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 other lines under salinity. In addition, 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 parameters analyzed (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  (μM g-1 FW) | LOX  (ΔAbs mg prot-1min-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 |
|  | LSD0.05 | 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 | \* | \*\*\* | \*\*\* |
|  | LSD0.05 | 8.65 | 0.85 | 0.09 |
| Analysis of variance | | |
| Salinity (S) |  | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | NS | \*\*\* | \*\*\* |
| S x M |  | \* | \*\*\* | \*\* |
| LSD0.05 |  | 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 in relation 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 O⁠2˙‾ concentration in all plants except in *BraA.cax1a-4* that presented similar values compared to control conditions. Salt stress caused a higher accumulation of H2O2 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 H2O2 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 in relation 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

with respect to R-o-18. With respect to 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 in relation to control conditions (Table 3).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | O⁠2˙‾ (μg g-1 FW) | H2O2  (μg g-1 FW) | SOD  (ΔAbs mg prot-1min-1) | CAT  (ΔAbs mg prot-1min-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 | \*\* | \*\*\* | \*\*\* | \*\* |
|  | LSD0.05 | 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 | \*\* | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 0.60 | 10.78 | 2.71 | 0.46 |
|  | |
| Salinity (S) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | NS | \*\*\* | \*\*\* | NS |
| S x M |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 |  | 0.34 | 6.32 | 1.74 | 0.33 |

**Table 3**. ROS (O⁠2˙‾ and H2O2) concentration and SOD and CAT activities in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

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 relation 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 in comparison to R-o-18 plants and similar increments (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 respect control. Reduced AsA showed a similar behavior as total AsA, but *BraA.cax1a-4* also accumulated more AsA in comparison to R-o-18. 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% with respect to controls. These results led to 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 GR activity whereas all *BraA.cax1a* plants showed a lower decrease as 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 for 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 | \*\*\* | \*\*\* | \*\*\* | \* |
|  | LSD0.05 | 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 | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 8.10 | 1.74 | 5.88 | 4.15 |
| Analysis of variance | | | | |  |
| Salinity (S) |  | \*\*\* | \*\*\* | NS | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| S x M |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 |  | 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 in relation 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 | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 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 | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 15.46 | 9.94 | 5.82 | 5.61 |
| Analysis of variance | | | | |  |
| Salinity (S) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| S x M |  | \*\*\* | \*\*\* | \*\*\* | \*\* |
| LSD0.05 |  | 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 in relation 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 in 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 while in the rest of the lines Chl-HSP70 levels remained substantially unchanged with respect 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 other lines showed a decrease respect 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 with respect 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. 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 Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ 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+ ratiocould also be related to a higher Ca2+ content in *BraA.cax1a-4* that could provide a better salt tolerance. Furthermore, *BraA.cax1a-4* mutation might enhance cytosolic Ca2+ concentration which activates stress responsesas observed in salt-tolerant maize genotypes (Zhao et al., 2019).In the roots, we observed an opposite trend: *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 ASK, Na which means that they favor Na+ uptake over K+. Although, both lines presented higher TSK, 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 used as indicators of lipid peroxidation caused by salinity as observed in several species analyzed (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 are indicative of membrane damage. In the present study, *B. rapa* showed increments for these parameters in plants grown under salinity, although *BraA.cax1a* plants presented lower values. Similar studies showed the relationship between Ca2+ and the protection of membranes against salinity (Bacha et al., 2015;Cantabella et al., 2017).In addition,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 since lower K+ levels usually are linked to membrane damages and K+ concentration can 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 O⁠2˙‾ and H2O2 concentrations are measured in plants grown under salinity (Sahin et al., 2018). ROS usually are more 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 O⁠2˙‾ and all *BraA.cax1a* also showed lower H2O2 levels especially *BraA.cax1a-4* plants. To neutralize ROS, plants activate antioxidant defenses such as SOD and CAT activities, enhanced under salinity. 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* plantsCAT 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 CO2 by photorespiration and thereby enhancing 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 O⁠2˙‾. These results highlight the importance of CAX1 transporter in the activation of antioxidant enzymes as observed in cax1 plants 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 in order 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 lower extent in *BraA.cax1a-4* plants that showed lower APX activity. APX activation probably increases H2O2 detoxification that is generated by salinity. However, *BraA.cax1a-4* could detoxify H2O2 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 in spite of the lower SOD activity. These results concurred 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 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 had a negative effect on GSH 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 are HSP70 that under abiotic stress binds to misfolded or truncated proteins protecting cells (Usman et al., 2017; Landi 2019). HSP70s have been proved to be key in salt-stress tolerance as silenced mutants for HSP70s showed inhibition of growth (Anaraki et al., 2018). In addition, the overexpression of HSP70 gene enhanced salinity tolerance(Augustine et al., 2015;Fu et al., 2016).Our experiments show that*BraA.cax1a* mutations affect HSP70s levels. Thus, all *BraA.cax1a* mutations showed higher Cyt-HSP70 accumulation which could be related to different cytosolic Ca2+ 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 response to salinity in barley plants. Thus, Cyt-HSP70 accumulated 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 Ca2+ 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 provide protection for the photosynthetic machinery in these lines under stress.

*4.7. Pro concentration*

Pro levels usually increase after the onset of saline stress and are one of the main indicators 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 O⁠2˙‾ (Gill and Tujeta, 2010). Accordingly, wenoteda 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 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. In addition, 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.

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