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

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**ABSTRACT**

Salinity is an important environmental factor that reduces plant productivity in many world regions. It affects negatively photosynthesis causing a growth reduction. Likewise,calcium (Ca2+) is crucial in plant stress response. Therefore, the modification of Ca2+ cation exchangers (CAX) transporters could be a potential strategy to increase plant tolerance to salinity. Using Targeting Induced Local Lesions in Genomes (TILLING), researchers generated three mutants of *Brassica rapa* CAX1a transporter. The aim of this study was to test the effect of those mutations on salt tolerance focusing on the response to the photosynthesis process. Thus,*BraA.cax1a* and R-o-18 plants were grown under salinity conditions, and parameters related to biomass, photosynthesis performance, glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), and soluble carbohydrates were measured. The results showed that *BraA.cax1a-4* mutation provided higher biomass and a better photosynthetic performance manifested by a higher water use efficiency (WUE), higher Fv/Fm, electron fluxes, and a higher Rubisco (EC 4.1.1.39) accumulation. In addition, *BraA.cax1a-4* presented increased osmotic protection through *myo*-inositol accumulation. On the other hand, *BraA.cax1a-7* produced some negative effects on photosynthesis performance and lower G6PDH and Rubisco accumulations. Therefore, this study points out *BraA.cax1a-4* as a useful mutation to improve photosynthetic performance in plants grown under saline conditions.

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

**Abbreviations**: CAX, cation exchangers; Chl, chlorophyll; G6PDH, glucose-6-phosphate dehydrogenase; PS, photosystem; TILLING, Targeting induced local lesions in genomes; WUE, water use efficiency

**1. Introduction**

One of the most pressing issues in the world, especially in the arid and semi-arid regions, influencing plant productivity is salinity. Higher concentrations of soluble salts either in soil or in irrigation water are the triggering factors of salinity. In recent years, this recurrent problem has caught the eye of many researchers, being predicted as the contributing factor in global warming [1]. Several crops i.e. cash, cereals, or forage crops among others are prone to this problem. Brassicaceae family including cabbage, broccoli, and rapeseed are also included in this affected group. According to the previous studies, a lot of these species are cultivated in areas with higher salinity and the ones that are on the verge of being saline [2]. Among the possible causes of salinity, ion toxicity, mainly of Na+, is the prominent one that interferes with vital processes within the plants [3].

One of the most important reasons for growth reduction in saline-stressed plants is that the photosynthesis process is altered either by the reduction in leaf area or by the photosynthesis imbalance process at a cellular level. Thus, salinity stress causes direct salt toxicity and triggers leaf senescence producing first chlorosis and finally leaf abscission [1]. In addition, the accumulation of salts in the growing medium reduces its osmotic potential, hindering water uptake by the roots [4]. Consequently, plants promote stomatal closure to reduce water loss. However, this closure limits CO2 diffusion inside the leaf and the limitation in CO2 supply inhibits the activity of the main enzyme for CO2 assimilation, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) [5]. Besides, the regeneration of reducing power by the Calvin-Benson-Bassham cycle is hindered, which produces an imbalance between the photosystem (PS) II activity and the electron flow. This imbalance leads to an excessive photochemical energy accumulation that must be dissipated as fluorescence or may generate reactive oxygen species (ROS) that in turn produce photodamage [6]. Furthermore, the excessive accumulation of Na+ ions has direct negative effects on the components of the photosynthetic machinery, limiting its performance [5].

Plants possess adaptation mechanisms to face salinity stress, including changes in ion homeostasis, water distribution, energy balance, and photosynthesis regulation. Plants regulate photosynthesis at different levels such as CO2 assimilation, electron transport, or pigments distribution [7,8].A good stomata regulation is crucial to maintain osmotic potential and avoid water loss by transpiration. Thus, plants with higher water use efficiency (WUE) present higher salt tolerance and a better stomatal aperture regulation to reduce water loss whereas maintaining an adequate CO2 flow into the leaf [9]. Another way to regulate osmotic potential is through the accumulation of osmolytes that decrease its value in the plant to allow water uptake. Examples of osmolytes are soluble carbohydrates such as glucose, sucrose, and inositol [1]. Moreover, another mechanism that contributes to salinity tolerance is glucose oxidation in the pentose phosphate pathway (OPPP). In OPPP, glucose-6-phosphate is oxidized to pentose-P, generating NADPH and supplying carbon skeletons for the synthesis of nucleotides, lignin, coenzymes, and amino acids. This process is driven by glucose-6-phosphate dehydrogenase enzyme (G6PDH, EC 1.1.1.49), whose activity is enhanced under stress conditions to regulate energy balance and ensure NADPH supply for stress defense mechanisms and CO2 assimilation [10]. Furthermore, the energy balance is also mediated by the distribution of components such as antenna pigments and other components of the electron transport chain of photosystems. The objective is to maximize energy uptake and transference whereas minimizing the energy losses in the form of heat or fluorescence [11].

Besides, another important element involved in photosynthesis regulation is calcium (Ca2+). Ca2+ is part of the oxygen-evolving complex and Ca2+ is necessary to activate electron transport chain proteins and Calvin-Benson-Bassham cycle enzymes [12]. Furthermore, Ca2+ fulfills crucial functions within plants such as maintaining membrane stability, osmotic balance and it is an important component in signaling processes. Indeed, Ca2+ ions act as signaling agents and their fluxes modulate plant responses to stress, regulating metabolic processes [13]. Likewise, Ca2+ is a Na+ antagonist and thereby Ca2+ has been considered crucial in plant adaptation to salinity [14]. Salinity as several other stress processes induces higher cytosolic Ca2+ concentration to trigger stress responses. Cation exchangers (CAX) transporters are crucial in the modulation of Ca2+ gradients in cells as they are necessary to restore normal Ca2+ levels [13]. It was observed different CAX activity in halophytes such as *Suaeda salsa*, which could be key in salt tolerance [15]. Hence, the generation of variations in the CAX transporter could be a potential strategy to increase plant tolerance to salinity [13].

TILLING (Targeting Induced Local Lesions in Genomes) technique has been used to generate modifications in CAX1 transporter. TILLING performs a screening of new variations in target genes that may be useful for the generation of plants with improved tolerance to stresses [16]. Through TILLING, researchers generated three mutants of *Brassica rapa* ssp. trilocularis ’R-o-18’ with modifications in CAX1a transporter; *BraA.CAX1a*: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12* [17]. These mutations shift AAs that could affect protein conformation and thus improve CAX1 function [18]. Therefore, the hypothesis to test is that *BraA.CAX1a* modifications have an effect on the tolerance of these mutants against salinity. In this work, we focus on the response to the photosynthesis process.

**2. Material and methods**

*2.1. Plant material and growth conditions*

The plant material used in this experiment was a parent line R-o-18 (without changes in BraA.CAX1a) of *B. rapa* ssp. trilocularis ’R-o-18’ and three different mutants for BraA.CAX1a transporter: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12*). The amino acidic variations produced in BraA.CAX1a transporter were: *BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56). Mutant plants were developed and identified as described by Lochlainn et al*.* [17] and Graham et al*.* [18]*.* The *B. rapa* seeds were germinated and placed in pots (13 cm x 13 cm x 12.5 cm) with vermiculite as substrate. Pots were placed in trays (55 cm x 40 cm x 8.5 cm). The trays with the pots were placed in a growth chamber with controlled environmental conditions: relative humidity 60-80%, temperature 22/18ºC (day/night), 14/10-h photoperiod, and 350 µmol m-2s-1 photosynthetically active radiation (PAR). Pots were watered with a nutritive solution composed of 4 mM KNO3, 4 mM Ca(NO3)2 • 4 H2O, 2 mM MgSO4 • 7 H2O, 6 mM KH2PO4, 1 mM NaH2PO4 • 2 H2O, 2 μM MnCl2 • 4 H2O, 1 µM ZnSO4, 0.25 μM CuSO4 • 5 H2O, 0.1 μM Na2MoO4 • 2 H2O, 5 µM Fe-chelate (Sequestrene; 138FeG100), and 10 µM H3BO3. The solution had a pH of 5.5–6.0 and was renewed every three days.

*2.2. Treatments and experimental design*

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

*2.3. Plant sampling*

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

*2.4. Pigment concentrations*

Leaves (0.1 g) were grounded and 1 ml of methanol was added for total chlorophyll (Chl) and carotenoid extraction. Then the mixture was centrifuged at 5000 × g for 5 min. Afterward, the absorbance of the supernatant was measured at three different wavelengths: 664, 648, and 470 nm. The Chl *a* and Chl *b* were assessed using the Lichtenthaler equation [19]. Total Chl was calculated as the sum of Chl *a* and Chl *b*.

*2.5. Fluorescence parameters*

Plant leaves were kept in the dark during 30 min using special leaf clip holders. Chl *a* fluorescence kinetics was registered using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King’s Lynn, Norfolk, UK); the fluorescence transients were induced by red light (650 nm) with 3000 µmol photons m-2s-1 light intensity. For each treatment, measurements were carried out in nine fully-expanded leaves at midstem position of different plants. Handy PEA software was used to obtain the values of all considered fluorescence parameters [20].

*2.6. G6PDH assay*

For G6PDH extraction, 300 mg of leaves were ground and suspended in 600 ml of a solution containing 50 mM Tris-HCl a pH 8.0, 10% glycerol, 15 mM NADP+, 5 mM MgCl2, 4 mM EDTA, and 1ml/30 gr Protease Inhibitor Cocktail (Sigma P9599). G6PDH activity was obtained as described previously by Castiglia et al. [21] registering NADP+ reduction at 340 nm using a Cary 60 spectrophotometer [Agilent Technologies, USA). The composition of the assay mixture was: the previously obtained extract, 50 mM Tris-HCl pH 8.0, 3 mM glucose-6P, 5 mM MgCl2, 150 μM NADP+. G6PDH activity was expressed as nmol NADPH min-1 mg-1 protein.

*2.7. Western blotting*

For Western blotting analysis, proteins were extracted following the same method as described by G6PDHassay. Then, proteins were separated by SDS-PAGE and transferred on a nitrocellulose membrane (Ge Healthcare) using the Transblot-turbo (Biorad, CA, Usa) as previously described [22]. Membranes were incubated with antibodies raised vs potato G6PDH isoforms [23]: cytosolic (Cyt-G6PDH), P1 (P1-G6PDH), and P2 (P2-G6PDH) and RuBISCO large subunit. After incubating the membrane with horseradish peroxidase (HRP)-linked secondary antibodies, cross-reacting polypeptides were identified by enhanced chemioluminescence (ECL) reaction (WesternBrightTM Quantum kit—Advansta, San Josè, CA, USA). The images were obtained by BioRad Chemidoc system (Bio-Rad, Hercules CA, USA). Control blot was performed using anti-tubulin antisera (Fig. S1).

*2.8. Leaf gas exchange parameters*

To register leaf gas exchange parameters an infra-red gas analyzer LICOR 6800 Portable Photosynthesis System (IRGA: LICOR Inc. Nebraska, USA) was used. Nine fully expanded leaves of nine different plants for each treatment were placed in a leaf cuvette set with optimal growth conditions. The instrument was warmed up for 30 min and calibrated before the measurements. Standard optimal cuvette conditions of 500 µmol mol⁻¹ CO2 concentration, 500 μmol m2 s-1 PAR, 60% relative humidity, and 30°C leaf temperature. Net photosynthesis rate (*A*), transpiration rate (*E*), stomatal conductance (*g*s), and intercellular CO2 (*C*i) were registered. Data were analyzed using the “Photosyn Assistant” software. Instantaneous water use efficiency (WUE) was calculated by dividing *A* by the corresponding *E*.

*2.9. Soluble Carbohydrates analysis*

Samples were lyophilized and soluble carbohydrates concentrations were determined in the Metabolomics Service of CEBAS-CSIC (Murcia, Spain) using 1H-NMR technique and according to the methodology described in Massaretto et al*.* (2018) [24].

*2.10. Statistical analysis*

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

**3. Results**

*3.1. Plant biomass*

The different *B. rapa* lines did not show significant differences in leaf DW under control conditions but the saline treatment caused a remarkable decrease in this parameter. Nevertheless, this reduction was lower in *BraA.cax1a-4* plants that presented higher leaf DW than the other lines (Fig. 1).



**Figure 1**. Effect of R-o-18 and *BraA.cax1a* mutations on leaf DW. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05). Asterisk (\*) indicates significative differences between control and 150 mM treatments.

*3.2. Photosynthetic pigments concentration*

Under control conditions, *BraA.cax1a* mutants showed lower total Chls concentration in comparison to R-o-18 plants. Furthermore, under saline conditions, the level of this parameter was lower in all lines compared to that of plants not supplied with NaCl. Comparing among lines, *BraA.cax1a-7* plants showed lower Chls levels than R-o-18, whereas the other mutants did not show significant differences in comparison to R-o-18 plants. However, *BraA.cax1a* plants presented higher Chl *a*/*b* ratios compared to R-o-18 plants. Like Chls, carotenoids were less concentrated in *BraA.cax1a* plants being *BraA.cax1a-7* the mutant with the lowest carotenoids concentration (Table 1).

**Table 1**. SPAD value, total Chls concentration, Chl a/b ratio and carotenoids concentration in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | Total Chls  mg g-1 FW | Chl *a*/*b* | Carotenoids  μg g-1 FW |
| Control | R-o-18 | 0.73a | 2.30b | 52.05a |
|  | *BraA.cax1a-4* | 0.67b | 2.37a | 49.10ab |
|  | *BraA.cax1a-7* | 0.61b | 2.24c | 49.34ab |
|  | *BraA.cax1a-12* | 0.63b | 2.31b | 45.88b |
|  | *p*-value | \*\* | \*\*\* | NS |
|  | LSD0.05 | 0.06 | 0.03 | 5.36 |
| 150 mM NaCl | R-o-18 | 0.63a | 2.17b | 52.19a |
| *BraA.cax1a-4* | 0.59a | 2.29a | 43.75b |
|  | *BraA.cax1a-7* | 0.53b | 2.29a | 39.54c |
|  | *BraA.cax1a-12* | 0.60a | 2.33a | 43.88b |
|  | *p*-value | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 0.05 | 0.05 | 3.82 |
| Analysis of variance | |  | |  |
| Salinity (S) |  | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\*\* | \*\*\* |
| S x M |  | NS | \*\*\* | \* |
| LSD0.05 |  | 0.03 | 0.03 | 3.19 |

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

*3.3. Chl a fluorescence parameters*

Comparing the different lines grown under control conditions, *BraA.cax1a-7* mutants registered higher Fo, Fm, and K step, and lower Fv/Fm and RC/ABS values than R-o-18 plants, whereas the rest of the lines showed similar values. Regarding salinity conditions, R-o-18 and *BraA.cax1a-12* plants showed higher Fo values, whereas all plants subjected to salinity presented lower Fm values in comparison to control conditions. Although *BraA.cax1a-7* plants registered the highest values for these parameters and *BraA.cax1a-4* plants presented the lowest Fo in comparison to the other lines. Salinity stress reduced the Fv/Fm values of all plants compared to those of plants not supplied with NaCl, but *BraA.cax1a-4* plants reached higher Fv/Fm values than the other lines. About ΨEo and ΦEo parameters, it was not observed significant effects of salinity nor mutations. Furthermore, salinity reduced PIABS value in all plants except in *BraA.cax1a-4* plants in comparison to control conditions but no different PIABS values were observed comparing among the four lines. Salinity reduced RC/ABS values in all *B. rapa* genotypes, whereas *BraA.cax1a-7* plants showed lower RC/ABS in comparison to R-o-18 plants. In contrast, salinity increased K step values in all the lines compared to plants that did not received NaCl, and *BraA.cax1a-7* reached the higher K step value compared to R-o-18 plants. In addition, NaCl application increased t Fm values of R-o-18 and *BraA.cax1a-7* plants in comparison to control conditions and all *BraA.cax1a* plants showed higher t Fm values compared to R-o-18 plants (Table 2).

**Table 2**. Photosynthetic fluorescence parameters in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Fo | Fm | Fv/Fm | ΨEo | ΦEo | PIABS | RC/ABS | K step | t Fm |
| Control | R-o-18 | 349b | 2203b | 0.841a | 0.66a | 0.56a | 9.82a | 1.00a | 460.8b | 700a |
|  | *BraA.cax1a-4* | 366b | 2232b | 0.836a | 0.66a | 0.55a | 8.61a | 0.94ab | 471.17b | 600a |
|  | *BraA.cax1a-7* | 465a | 2579a | 0.821b | 0.65a | 0.51a | 8.99a | 0.87b | 523.33a | 700a |
|  | *BraA.cax1a-12* | 351b | 2207b | 0.841a | 0.62a | 0.52a | 9.61a | 0.96a | 493.33ab | 660a |
|  | *p*-value | \*\* | \* | \*\*\* | NS | NS | NS | \*\* | \* | NS |
|  | LSD0.05 | 67 | 282 | 0.012 | 0.07 | 0.07 | 1.37 | 0.08 | 41.03 | 118 |
| 150 mM NaCl | R-o-18 | 395bc | 1937ab | 0.796b | 0.68a | 0.56a | 8.76a | 0.90a | 524.83b | 394b |
| *BraA.cax1a-4* | 359c | 1828b | 0.804a | 0.66a | 0.55a | 8.56a | 0.88a | 584.67ab | 575a |
|  | *BraA.cax1a-7* | 417a | 2063a | 0.798ab | 0.68a | 0.56a | 7.84a | 0.69b | 671.50a | 580a |
|  | *BraA.cax1a-12* | 393ab | 1966ab | 0.801ab | 0.65a | 0.53a | 8.68a | 0.81a | 582.17ab | 620a |
|  | *p*-value | \*\* | \* | \* | NS | NS | NS | \*\*\* | \* | \*\*\* |
|  | LSD0.05 | 30 | 142 | 0.006 | 0.06 | 0.05 | 1.56 | 0.09 | 114.4 | 87 |
| Analysis of variance | | | | |  |  |  |  |  |  |
| Salinity (S) |  | \*\*\* | \*\*\* | \*\*\* | NS | NS | \* | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\* | \*\*\* | NS | NS | NS | \*\*\* | \*\* | \* |
| S x M |  | NS | NS | \*\*\* | NS | NS | NS | NS | NS | \*\*\* |
| LSD0.05 |  | 35 | 153 | 0.007 | 0.05 | 0.04 | 0.98 | 0.06 | 58.87 | 71 |

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

Regarding electron fluxes, *BraA.cax1a* plants presented higher ABS/RC, TRo/RC, and ETo/RC values, and *BraA.cax1a-7* plants registered higher DIo/RC value in comparison to the R-o-18 line when they were grown without NaCl. Under saline conditions, all lines showed a similar decrease caused by salinity in ABS/RC, TRo/RC, and ETo/RC parameters in comparison to control conditions, although *BraA.cax1a* mutants register higher values than R-o-18 plants (Fig. 2A-C). Besides, salinity increase DIo/RC value in *BraA.cax1a-4* and especially in the *BraA.cax1a-7* line compared to plants without NaCl. Indeed, under saline conditions, *BraA.cax1a-7* registered the highest DIo/RC value in comparison to the other lines (Fig. 2D).



**Figure 2**. Effect of R-o-18 and *BraA.cax1a* mutations on photosynthetic electron fluxes. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05). Asterisk (\*) indicates significative differences between control and 150 mM treatments.

*3.4. G6PDH and Rubisco*

Salinity did not affect G6PDH activity in *B. rapa* plants except in *BraA.cax1a-7* plants that showed an increment in comparison to plants not supplied with NaCl. However, this mutant showed the lowest G6PDH activity, whereas *BraA.cax1a-4* reached the highest G6PDH activity compared to the other lines grown under both saline and control conditions (Fig. 3). Furthermore, western blot analysis showed lower Cyt-G6PDH and Rubisco accumulations in the *BraA.cax1a-7* lineunder control conditions. R-o-18 and *BraA.cax1a-4* plants showed a reduction in Cyt-G6PDH and Rubisco presence caused by NaCl, whereas in the *BraA.cax1a-7* and *BraA.cax1a-12* lines the accumulation of these two elements increased compared to plants not grown under salinity. Comparing among lines, *BraA.cax1a-4* and *BraA.cax1a-12* plants showed higher Cyt-G6PDH and Rubisco accumulations, whereas *BraA.cax1a-7* registered lower accumulation of these enzymes compared to R-o-18 plants under saline conditions (Fig. 4A and 4D). With reference to P1-G6PDH isoform, all *BraA.cax1a* plants showed higher accumulation than R-o-18 plants under no salinity conditions. Nevertheless, P1-G6PDH accumulation was reduced by salinity in *BraA.cax1a* plants compared to control conditions. The presence of this isoform was lower in *BraA.cax1a-4* but higher in *BraA.cax1a-7* and *BraA.cax1a-12* plants in comparison to R-o-18 plants (Fig. 4B). Besides, *BraA.cax1a-4* showed lower accumulation of P2-G6PDH isoform, whereas *BraA.cax1a-7* and *BraA.cax1a-12* plants did not show significant changes compared to control conditions. Comparing among genotypes, *BraA.cax1a-4* showed higher P2-G6PDH, whereas the presence of this isoform was lower in *BraA.cax1a-7* compared to R-o-18 plants.



**Figure 3**. Effect of R-o-18 and *BraA.cax1a* mutations on G6PDH activity. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05). Asterisk (\*) indicates significative differences between control and 150 mM treatments.



**Figure 4**. Effect of R-o-18 and *BraA.cax1a* mutations on the accumulation of G6PDH and RuBisCO. The upper panel shows western blotting. Lower panel shows densitometric analysis using Image J software.

*3.5. Gas exchange parameters*

In control plants, no differences among lines were observed regarding *A*, whereas the *BraA.cax1a-12* line registered higher *E*, *g*s, and *C*i and lower WUE compared to R-o-18 plants. Moreover, NaCl application reduced *A*, *E*, *g*s in *B. rapa* plants in comparison to control conditions. Comparing among lines, *BraA.cax1a-4* and *BraA.cax1a-7* showed higher *A*, *E*, and *g*s than R-o-18 plants.Regarding *C*i, no significant effects of salinity were observed in *BraA.cax1a* plants compared to control conditions and no differences between lines grown under salinity were observed. In addition, water use efficiency (WUE) was lower in all the lines subjected to salinity, except *BraA.cax1a-12*, in comparison to plants not supplied with NaCl. Furthermore, *BraA.cax1a-4* plants registered higher WUE compared to the other lines (Table 3).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | *A* (µmol m⁻² s⁻¹) | *E* (mmol m⁻² s⁻¹) | *g*s (mol m⁻² s⁻¹) | *C*i  (µmol mol⁻¹) | WUE (μmol mmol-1) |
| Control | R-o-18 | 8.99a | 2.89b | 0.18b | 375b | 3.15a |
|  | *BraA.cax1a-4* | 9.20a | 3.15b | 0.24b | 402ab | 2.96ab |
|  | *BraA.cax1a-7* | 8.88a | 4.33ab | 0.35ab | 428a | 2.42b |
|  | *BraA.cax1a-12* | 8.80a | 5.69a | 0.47a | 419a | 1.31c |
|  | *p*-value | NS | \* | \* | \* | \*\*\* |
|  | LSD0.05 | 1.51 | 2.23 | 0.21 | 39.93 | 0.55 |
| 150 mM NaCl | R-o-18 | 1.35b | 0.60c | 0.04c | 431a | 1.66b |
| *BraA.cax1a-4* | 2.16a | 0.77ab | 0.05ab | 418a | 2.11a |
|  | *BraA.cax1a-7* | 2.02a | 1.06a | 0.07a | 426a | 1.62b |
|  | *BraA.cax1a-12* | 1.77ab | 0.88bc | 0.04bc | 406a | 2.02ab |
|  | *p*-value | \* | \* | \*\* | NS | \* |
|  | LSD0.05 | 0.67 | 0.27 | 0.01 | 38.26 | 0.42 |
| Analysis of variance | | | | |  |  |
| Salinity (S) |  | \*\*\* | \*\*\* | \*\*\* | NS | \*\*\* |
| Mutation (M) |  | \* | \* | \* | NS | \*\*\* |
| S x M |  | NS | NS | \* | NS | \*\*\* |
| LSD0.05 |  | 0.78 | 1.06 | 0.1 | 26.19 | 0.34 |

**Table 3**. Gas exchange parameters 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 levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.6. Soluble carbohydrates concentration*

*BraA.cax1a-7* plants presented lower glucose, fructose, and sucrose concentrations, whereas *BraA.cax1a-4* and *BraA.cax1a-*12 registered lower *myo*-inositol concentration compared to R-o-18 plants when NaCl was not applied. Regardless of the genotype, salinity caused an increase in all the analyzed carbohydrates except sucrose in comparison to control conditions. Comparing among lines, *BraA.cax1a* plants showed lower glucose and *myo*-inositol concentrations in comparison to R-o-18 plants, highlighting *BraA.cax1a-7* plants that presented the lowest values. Besides, *BraA.cax1a-4* and specially *BraA.cax1a-7* plants registered lower fructose concentration compared to R-o-18 plants. However, *BraA.cax1a-7* was the unique line presenting measurable levels of sucrose under saline conditions (Table 4).

**Table 4.** Carbohydrates concentration in R-o-18 and *BraA.cax1a* mutants grown under control and salinity conditions

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | Glucose  mg g-1 DW | Fructose  mg g-1 DW | Sucrose (mg g-1 DW) | *Myo*-inositol (mg g-1 DW) |
| Control | R-o-18 | 1.74b | 5.36b | 0.12b | 2.62a |
|  | *BraA.cax1a-4* | 1.14c | 7.38a | 0.13b | 1.92c |
|  | *BraA.cax1a-7* | 1.22c | 4.56c | 0.10c | 2.49a |
|  | *BraA.cax1a-12* | 2.03a | 4.36c | 0.31a | 2.16b |
|  | *p*-value | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 0.15 | 0.52 | 0.02 | 0.22 |
| 150 mM NaCl | R-o-18 | 7.17a | 16.74a | nd | 6.16a |
| *BraA.cax1a-4* | 2.78c | 12.46b | nd | 4.97b |
|  | *BraA.cax1a-7* | 1.71d | 8.84c | 0.03 | 4.45c |
|  | *BraA.cax1a-12* | 4.99b | 17.85a | nd | 5.27b |
|  | *p*-value | \*\*\* | \*\*\* |  | \*\*\* |
|  | LSD0.05 | 0.44 | 1.36 |  | 0.41 |
| Analysis of variance | | | | |  |
| Salinity (S) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| S x M |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 |  | 0.21 | 0.67 | 0.01 | 0.21 |

Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values below the detection limit are indicated as nd. Values with different letters indicate significant differences. The level of significance were represented by p<0.001 (\*\*\*).

**4. Discussion**

The most immediate effect of salinity on plants and specifically in *Brassica* crops is growth reduction due to it is considered such as species with moderate sensitivity to saline stress [2]. Thus, in this present experiment, R-o-18 plants grown under saline conditions showed a 73% biomass decrease. However, this reduction was significantly lesser in *BraA.cax1a-4* plants, which presented higher shoot biomass in comparison to the other lines. Thereby, *BraA.cax1a-4* mutation could provide greater tolerance to high NaCl concentration in the growth medium.

Several experiments showed that salinity also causes a reduction in the concentration of photosynthetic pigments in plants [1,25,26]. Actually, the chlorophyll content decreases more in salt-sensitive species, whereas it can even increase in salt-tolerant species [1,3]. In the present experiment, all *B. rapa* lines showed a decrease in total Chls concentration caused by salinity. Although this decrease in Chls concentration was lower in *BraA.cax1a-12* plants, this mutation did not provide higher salinity tolerance. Furthermore, other studies observed that Ca application enhances Chl *a* concentration in plants grown under saline conditions [27,28]. Nevertheless, *BraA.cax1a*-7 had negative effect on Chl concentration, although it was not determinant for lower biomass because this mutant showed a similar biomass loss than the other lines.

Stresses such as salinity usually increase the proportion of Chl *b* over Chl *a* leading to a lower Chl *a*/*b* ratio [26]. In chloroplast, Chl *a* is principally presented in reactive centers (RCs) and core antenna proteins of PS II, whereas Chl *b* is located mainly in light-harvesting complexes. A higher Chl *a*/*b* ratio usually is indicative of the conversion of Chl *b* into Chl *a* to maintain active RCs under stress [29,30]. This conversion could be the cause of the higher Chl *a*/*b* ratio observed in *BraA.cax1a* mutants. Alternatively, carotenoids act as accessory pigments, light-harvesting compounds, and as antioxidants [6]. Regarding carotenoids, other experiments measured either no effects or a decrease in their concentrations under saline conditions [25]. However,in this study salinity only produced a clear reduction in *BraA.cax1a-7* plants, although all *BraA.cax1a* reduced its concentration. Hence, changes in Ca2+ fluxes presumably induced by *BraA.cax1a* mutations could influence the distribution of leaf photosynthetic pigments in plants subjected to salinity, in a non-determinant way on growth.

Photosynthetic pigments, in addition to transferring useful energy for photosynthesis, emit back as fluorescence a part of the energy absorbed. This fluorescence emission is higher under stress conditions. Thus, Chl *a* fluorescence is a good method to evaluate PS II performance and the alterations in photosynthesis caused by stress [31]. Derived from Chl *a* fluorescence analysis, Fv/Fm and PIABS parameters are reliable indicators of PS II performance that usually are reduced in plants grown under saline stress [11,32]. Indeed, a decrease in these parameters was observed in another experiment in *B. rapa* plants grown with 200 mM NaCl, although this reduction was not produced when 100 mM NaCl was applied [2].In the present experiment, using 150 mM NaCl, we observed a reduction in Fv/Fm and PIABS parameters and also negative effects were produced on other fluorescence parameters such as RC/ABS, K step, and electron fluxes caused by salinity, which indicates an impairment in energy transference within photosystems [33]. Comparing lines, *BraA.cax1a-4* showed better photosynthetic performance because registered higher Fv/Fm and electron fluxes values. Although *BraA.cax1a-7* also presented higher fluxes, it showed the highest DIo/RC value, which suggests a higher wasting of photosynthetic energy. Furthermore, this mutant showed lower RC/ABS and higher K step values suggesting a worse photosynthetic performance. Zhao et al.[34]study compared maize genotypes with different salt tolerance and observed that differences in CAX1 transporter activity provided different PS performance, which could explain the differences observed in *BraA.cax1a* mutants.

Another process related to energy supply is OPPP in which glucose-6-phosphate (G6P) is oxidized to pentose-P, generating NADPH. The key enzyme in this process is G6PDH [10]. In salt-stress conditions, G6PDH activity is enhanced to supply the demand for reductants for antioxidant systems and other stress responses [35]. In contrast, this induction of G6PDH activity was not observed as a result of salinity stress in our experiment. However, *BraA.cax1a-4* plants showed the highest G6PDH activity under salinity conditions, which could contribute to a higher NADPH generation to face oxidative stress. On the other hand, the lower G6PDH activity in *BraA.cax1a-7*might cause a NADPH shortage, thereby explaining its worse photosynthetic performance.

G6PDH enzyme has three main isoforms in plants. The Cyt-G6PDH isoform represents about 60-85% of all G6PDH activity. The other isoforms are P1-G6PDH (chloroplastic isoform) and P2-G6PDHs (plastidic isoform). Considering these isoforms, Cyt-G6PDH accumulation was well correlated with G6PDH activity agreeing with the fact that cytosolic isoform is responsible for the main G6PDH activity in cells. Alternatively, P1-G6PDH functions in the dark to generate NADPH to maintain basal C metabolism [10].In *B. rapa* plants grown under salinity, P1-G6PDH accumulation showed a particular pattern because *BraA.cax1a-7* showed the highest P1-G6PDH probably to offset its lower photosynthesis performance and to maintain NADPH generation in chloroplasts. Interestingly, under control conditions in *BraA.cax1a-7* the increased occurrence of P1-G6PDH corresponds to lower levels of P2-G6PDH, suggesting a compensation effect; on the other hand, salinity increased specifically the plastidic P2-G6PDH, supporting the hypothesis that this isoform is able to sustain the increased requirement of reductants under stress [35,36].

An adequate NADPH supply is curcial for optimal plant funcitioning and to avoid imbalances between photochemical phase of photosynthesis and Calvin-Benson-Bassham cycle. The main enzyme in this cycle is Rubisco, which is a key enzyme in photosynthesis that fixes CO2 in plants and its activity is regulated under stress occurrence [5]. Thus, concentrations above 100 mM NaCl reduce Rubisco activity and accumulation. Furthermore, its activity is closely related to the availability of CO2 in photosynthetic tissues [37,38].NaCl could difficult CO2 diffusion in mesophyll regardless of stomata closure and thereby reduce Rubisco activity [25].However,in the present study, Rubisco accumulation was not correlated with *C*i plants subjected to salinity. Therefore, the difference in Rubisco accumulation observed between the different lines might be related to the effect of Ca2+ regulation. Thus, Ca2+ signaling is crucial for the enzyme activities of Calvin-Benson-Bassham such as Rubisco [12]. Similar to other photosynthetic parameters, *BraA.cax1a-4* showed higher Rubisco accumulation and this enzyme was less accumulated in *BraA.cax1a-7* plants. Higher Rubisco availability could be key to sustain the higher biomass observed in *BraA.cax1a-4* plants grown under saline conditions.

It is necessary a good CO2 supply for optimal Rubisco activity. The gas exchange parameters estimate CO2 and H2O fluxes and thereby they are useful to evaluate the photosynthesis and also the transpiration processes in photosynthetic organisms. Likewise, changes in leaf gas exchanger parameters are good indicators of stress in plants. For instance, salinity usually reduces the values of these parameters in plants [5]. However, several authors observed that Ca application restored gas exchange values, which was correlated with a growth improvement [27,28,39]. We observed that salinity reduced the values of gas exchange parameters of all the analyzed plants but to a lesser extent in *BraA.cax1a-4* and *BraA.cax1a-7* plants. Indeed, these plants presented higher *A* than R-o-18, although this increment only was translated in higher biomass in *BraA.cax1a-4* plants. Probably, other factors such as the higher water loss by transpiration, reflected in higher *E* and *g*s values, and the lower WUE influenced negatively to *BraA.cax1a-7* plants.Despite *BraA.cax1a-4* plants also showed higher E and *g*s values, they presented a greater WUE value, which suggests a better stomata regulation to avoid excessive water loss but maintaining CO2 capture. Ca2+ is involved in stomatal response [26], which may explain the effect of salinity and *BraA.cax1a* mutations on *g*s and transpiration.

Soluble carbohydrates, besides being the direct result of the photosynthetic process, generally are accumulated in plants as a consequence of salt stress. Thus, compounds such as glucose, fructose, sucrose, and *myo*-inositol act as osmoprotectors, carbon storage, and also radical scavenging [5,39]. The analysis of soluble carbohydrates in *B. rapa* plants showed an increase in their concentrations caused by salinity. Furthermore, Ca2+ is related to soluble carbohydrates accumulation and the enzyme activities for their synthesis and catabolism [12,40]. This fact could explain the lower soluble carbohydrates concentration observed in *BraA.cax1a* plants. Among them, *BraA.cax1a-7* plants showed the lowest monosaccharides concentrations, which might be caused by a lower carbohydrate synthesis derived from its worse photosynthetic performance. However, the higher sucrose concentration in *BraA.cax1a-7* plants might be related to higher carbohydrate remobilization, being that sucrose fulfills this function in stressed plants [41]. Regarding *myo*-inositol, it plays an important role in salt stress response acting as an osmolyte and is accumulated in halophytes [42]. In addition, the increment in *myo*-inositol levels in salt-stressed plants has proved to be an effective strategy to provide higher salt tolerance [43,44]. Thus, in the present study, the 159% increment of myo-inositol concentration in *BraA.cax1a-4* plants could be key to the higher tolerance observed in this line.

**5. Conclusion**

In conclusion, salinity application caused stress to *B. rapa* plants manifested by a decrease in biomass and a negative effect on photosynthetic performance. However, *BraA.cax1a* mutations influenced *B. rapa* response to salinity. Indeed, *BraA.cax1a-4* mutation provided higher biomass and better photosynthetic performance. Thus, these plants showed better stomata regulation, electron fluxes, and higher Rubisco accumulation. In addition, *BraA.cax1a-4* could present higher NADPH production through G6PDH activity and greater osmotic protection through *myo*-inositol accumulation. On the other hand, *BraA.cax1a-7* mutation produces negative effects on photosynthesis performance, such as a higher energy loss through fluorescence and a worse electron transport efficiency and lower G6PDH activity, and Rubisco and monosaccharides accumulations. Therefore, this study points out *BraA.cax1a-4* as a useful mutation to improve photosynthetic performance in plants grown under saline conditions.

**Author contributions**

Conceptualization S.E. and B.B; Methodology, E.N.L., V.P., F.J.L.-M., and J.J.R.; Validation, S.E., and B.B; Formal analysis, E.N.L. and V.P.; E.N.L., V.P, Data curation, E.N.L., V.P., F.J.L.-M., and J.J.R.; Writing—original draft preparation, E.N.L, S.E., and B.B.; Writing—review and editing, S.E., and B.B. All authors have read and agreed to the published version of the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Fig. S1. Result of control blot using anti-tubulin antisera