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

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ABSTRACT

Calcium (Ca) is an essential macronutrient for plants and its homeostasis is basic for many processes in plants. Therefore, both Ca deficiency and toxicity constitute potential issues for crops. CAX1 transporter is a potential target to obtain plants with better Ca homeostasis and higher Ca concentration in edible parts. Three *Brassica rapa* mutants for CAX1 were obtained through TILLING. The objective of this work is to evaluate the growth, physiological state and nutrients concentration of these mutants grown with different Ca doses. The mutants and the parental line were grown under low, control and high Ca doses and parameters related to their oxidative stress, photosynthetic performance and nutrients concentration were determined. *BraA.cax1a-4* and *BraA.cax1a-7* mutants presented lower total Chl, an altered photosynthesis performance and higher ROS levels. *BraA.cax1a-12* mutant grew better under high Ca conditions. All mutants accumulated more Ca and Mg in leaves under control and high Ca doses and accumulated more Fe regardless the Ca dose. The results obtained point to *BraA.cax1a-12* as a potential candidate for biofortification with Fe, Ca and Mg since it accumulate higher concentrations of these elements, do not present an altered growth and is able to tolerate higher Ca doses.

1. Introduction

Calcium (Ca) is an essential macronutrient for plants that is present in membranes and cell walls playing a basic structure role, and in the cytosol being crucial in cell signalling processes [1]. For this reason, Ca deficiency produces serious alterations in plants and it may cost losses in crop productions. Ca deficiency can occur even whether there is an adequate supply due to low redistribution or limitations in its transport [1,2]. On the other side, Ca toxicity also reduces the plant growth rate and produce damages due to the formation of Ca oxalate crystals. Cytosolic Ca concentration must be maintained at submicromolar levels in the resting cell in order to allow rapid increases for cell signalling, which can be jeopardized by Ca toxicity [1]. Ca fluxes are also necessary in cell guards for stomata closure, so an elevated Ca concentration may promote this closure and thereby a reduction in internal CO_2 concentration and a lower photosynthesis rate [2]. Both Ca deficiency and toxicity, constitute abiotic stresses that interfere with photosynthesis impairing electron transport, decreasing photosystems efficiency, reducing photosynthetic pigments, and promoting the formation of reactive oxygen species (ROS) [3]. In turn, ROS damage the photosynthetic apparatus through the disruption of thylakoid structures, inhibition of chloroplastic enzymes, and blocking PSII repair process [4].

Plants prevent Ca disorders through the regulation of plant cation/H^+ exchangers (CAXs) [2]. CAXs are a family of Ca/H antiporters located on plasma and organelle membranes including vacuoles. Together with Ca-ATPases, CAXs are responsible of Ca homeostasis and Ca removal from the cytosol to generate different Ca profiles to respond environmental cues or in signalling processes [5]. CAXs are involved in several important aspects of plant growth and development playing a role in stomatal conductance and in pH regulation [6]. There is a strong correlation between CAXs expression and Ca accumulation. Thus, *Brassica rapa* plants have an enhanced Ca accumulation in palisade mesophyll cells [7] where CAX expression is higher [5]. Among CAXs, CAX1 is one of the antiporters with greater Ca/H activity [6]. *CAX1* was identified as an expression quantitative trait loci that is affected by external Ca concentration in *B. rapa* [8]. Therefore, CAX1 is a potential target to obtain plants with better Ca homeostasis or with higher Ca concentrations in edible parts [6]. This fact could be useful since Ca, as well as iron (Fe) and magnesium (Mg) are essential elements for human diet. Stein [9] reported that two-thirds of the human population have a deficient diet of at least one of these elements, increasing the risk of

Abbreviations: APX, ascorbate peroxidase; CaUpE, Ca uptake efficiency; CaUtE, Ca utilisation efficiency; CAX, cation/H^+ exchanger; Chl, chlorophyll; DC, distribution coefficient; GMOs, genetically modified organisms; LOX, lipoxygenase; MDA, malondialdehyde; ROS, reactive oxygen species; TILLING, targeting induced local lesions in genomes

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

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

Values are means (n = 9) and differences between means were compared by Fisher's least-significance test (LSD; P = .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 $p < 0.001$ (***).

certain diseases. Crop nutrients can be improved through two ways: providing an adequate nutrient supply in the culture medium, considering interaction between nutrients, and the other way is through crop breeding (traditional breeding and by the use of genetically modified organisms (GMOs)) [10]. Experiments have already been carried out in this regard through the expression of *Arabidopsis thaliana CAX1* (*AtCAX1*) that increased Ca concentration in vegetables such as carrots [11], and lettuce [12]. These vegetables are GMOs, i.e. they were obtained by genetic engineering techniques. A possible alternative to the use of GMOs would be the generation of mutants with a modification in CAX1 activity affecting its self-regulation or activity.

A successful approach to obtain CAX1 variants is TILLING (Targeting Induced Local Lesions In Genomes). TILLING make possible the generation and the study of allelic series of mutations in order to evaluate their effects on gene expression and in protein structure and function [8]. TILLING was used to generate and identify three missense mutations in *B. rapa* ssp. trilocularis 'R-o-18' Ca transporter; *BraA.CAX1a*: *BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56) [13]. These mutations affect AAs upstream of the N-terminal autoinhibitory domain, but that could change protein conformation and thereby affecting CAX1 function or activity [8]. The genotyping and characterization of these mutants has been started. *BraA.cax1a-4* and *BraA.cax1a-7* lines presented paler/yellow leaves than parental line R-o-18 and in *BraA.cax1a-7* and *BraA.cax1a-12* lines was detected a variation in Ca concentration with respect their segregant wild types [8]. The species chosen for this study presents a rapid cycle, is self-compatible and include vegetable crops such as Chinese cabbage, turnip and some oil-seed crops [13]. Therefore, the working hypothesis to test is that CAX1a mutations will cause changes in growth, physiological state and nutrients accumulation and these changes will be influenced by Ca dose applied. The results could be useful to make an initial evaluation in order to improve *B. rapa* and other related crop species.

2. Material and methods

2.1. Plant material, growth conditions, and treatments

Three *B. rapa* ssp. trilocularis 'R-o-18' mutants (*BraA.CAX1a*: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12*) and the parent line R-o-18 were employed as plant material for the experiment [13]. Seeds were sown on filter paper moistened with milli-Q water (18.2 MV cm) in 9 cm Petri dishes. The dishes were sealed with plastic film, and incubated in the dark for 1 d at 4 °C 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 22/18 °C (day/night) and 14/10-h photoperiod at a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Throughout the experiment the plants received a growth solution composed of 4 mM KNO_3, 3 mM NH_4NO_3, 2 mM $MgSO_4{\cdot}7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 2 µM $MnCl_2{\cdot}4H_2O$, 0.25 µM $CuSO_4{\cdot}5H_2O$, 0.1 µM $Na_2MoO_4{\cdot}2H_2O$, 5 µM Fe-chelate (Sequestrene; 138FeG100) and 10 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was renewed every three days.

2.2. Experimental design and treatments

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

2.3. Plant sampling

Fully expanded leaves were washed with distilled water, dried on filter paper, and weighed for fresh weight (FW). Half of the leaves from each treatment were frozen at −30 °C for later biochemical assays and