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Ectopic expression of a maize calreticulin mitigates calcium deficiency-like disorders in *sCAX1*-expressing tobacco and tomato

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Abstract

Deregulated expression of an *Arabidopsis* H^+/Ca^{2+} antiporter (sCAX1) in agricultural crops increases total calcium (Ca^{2+}) but may result in yield losses due to Ca^{2+} deficiency-like symptoms. Here we demonstrate that co-expression of a maize calreticulin (CRT, a Ca^{2+} binding protein located at endoplasmic reticulum) in *sCAX1*-expressing tobacco and tomato plants mitigated these adverse effects while maintaining enhanced Ca^{2+} content. Co-expression of *CRT* and *sCAX1* could alleviate the hypersensitivity to ion imbalance in tobacco plants. Furthermore, blossom-end rot (BER) in tomato may be linked to changes in CAX activity and enhanced *CRT* expression mitigated BER in *sCAX1* expressing lines. These findings suggest that co-expressing Ca^{2+} transporters and binding proteins at different intracellular compartments can alter the content and distribution of Ca^{2+} within the plant matrix.

Key words: Calcium CAX CRT Co-expression Tomato

1 **Introduction**

2

3 In vegetables and fruits, calcium (Ca^{2+}) deficiency is a critical factor reducing their
4 quality and yield due to Ca^{2+}-related physiological disorders, such as blossom-end rot
5 (BER) in tomato, pepper, eggplant and melon, tipburn in lettuce, celery and cabbage,
6 and bitter pit in apple fruit (White and Broadley 2003; Dayod et al. 2010; de Freitas et
7 al. 2011). Moreover, low human dietary intake of Ca^{2+} has been associated with a
8 disease, osteoporosis, which may lead to a bone fracture (Bachrach 2001). Plant-based
9 foods are good sources of dietary Ca^{2+}; however, increased amounts in particular
10 foods may help ameliorate the incidence of osteoporosis caused by consumption of
11 inadequate dietary Ca^{2+} (Hirschi 2009; Park et al. 2009). Therefore, a better
12 understanding of Ca^{2+} improvement in plant cells is required in order to positively
13 impact human nutrition and improve fruit and vegetable production.

14 Calcium is unique amongst the elements in plants and animals because it plays
15 both a pivotal structural and, an essential, signaling role (White and Broadley 2003;
16 Hirschi 2004). Consequently steep gradients for Ca^{2+} exist across cell membranes
17 and cell endomembranes: the plasma membrane (PM), tonoplast (TN), and the
18 endoplasmic reticulum (ER). Gradients across these organelles are important for
19 normal cellular function and for the regulation of metabolic processes which requires
20 punctilious regulation of cytosolic Ca^{2+}. These gradients are established by a
21 dynamic balance between influx and efflux of Ca^{2+} across each of the cellular
22 membranes.

1 The concentration gradient of Ca^{2+} across the TN is established partially by
2 high-capacity H^+/Ca^{2+} antiporters (Zhao et al. 2009). Among them, CAXs (Cation/H^+
3 exchangers), a group of high-capacity, low-affinity transporters that export cations out
4 of the cytosol to maintain ion homeostasis across biological membranes (Pittman and
5 Hirschi 2003), have been physiologically characterized from a variety of plants. The
6 first *Arabidopsis CAX* gene, *CAX1* was identified by its ability to suppress the Ca^{2+}
7 sensitivity of a yeast mutant deleted in vacuolar Ca^{2+} transport (Hirschi et al. 1996).
8 CAX1 contains an additional 36 amino acid at its N-terminus that reduces the
9 transport activity in both yeast and plant expression assays (Pittman and Hirschi 2001;
10 Mei et al. 2007). When the N-terminal truncated version (sCAX1) is ectopically
11 expressed in potato, carrot and lettuce, Ca^{2+} content in their edible tissues increases
12 (Park et al. 2005b; Park et al. 2009). However, in some cases, these changes also
13 produce deleterious phenotypes that impact yield (Hirschi 1999; Park et al. 2005a).
14 Tempering expression of *sCAX1* driven by a different promoter results in healthier
15 plants but they often accumulate less Ca^{2+} (Park *et al.*, 2005a).
16 Tobacco lines expressing *sCAX1* increase Ca^{2+} content in their tissues, but also
17 display severe Ca^{2+} deficiency-like symptoms, such as apical leaf tip burning and
18 sensitivity to ion imbalances (Hirschi 1999). In addition, while the fruits of
19 s*CAX1*-expressing tomato plants accumulate higher total Ca^{2+} than vector control
20 plants, the *sCAX1*-expressing tomatoes show increased incidence of distinct necrotic
21 lesions in the distal portion of fruits, termed blossom-end rot (BER), which is
22 presumed to be caused by aberrant Ca^{2+} homeostasis in fruit cells (Park et al. 2005a).

1 These phenomena are an obstacle for the development of Ca^{2+}-biofortified crops.

2 Our working hypothesis is that the increased expression of *sCAX1* in
3 conjunction with Ca^{2+} binding proteins on another endomembrane may reduce these
4 deleterious phenotypes. Calreticulin (CRT), a Ca^{2+}-binding protein mainly resident in
5 the ER, has been known as an effective Ca^{2+} buffer protein that may allow the
6 transient storage of Ca^{2+} and play a role in stress responses (Jia et al. 2009).
7 Over-expression of a maize *CRT* cDNA in tobacco suspension cells results in a
8 two-fold increase in Ca^{2+} accumulation in the ER-enriched fraction *in vitro* (Persson
9 et al. 2001) and could improve growth of tobacco cell suspensions in high-Ca^{2+}
10 medium (Akesson et al. 2005).

11 Here, we express a maize *CRT* in *sCAX1*-expressing tobacco and tomato
12 plants to test our hypothesis if the expression of *CRT* gene can mitigate Ca^{2+}-related
13 cellular dysfunction resulted from expressing of *sCAX1* in tobacco and tomato plants
14 while maintaining enhanced Ca^{2+} content. Our findings suggest that co-expressing
15 transporters and binding-proteins may be a means of boosting plant nutrient content
16 without adversely affecting yield. To our knowledge, this study represents the first
17 attempts to increase the Ca^{2+} content of plants using co-expression of two genes
18 which play important roles in the regulation of Ca^{2+}.

19

20 Materials and Methods

21

22 Bacterial strain and plasmid

1 The pCaMV::sCAX1 [*sCAX1* driven by the cauliflower mosaic virus (CaMV) 35S
2 promoter] expression vector was previously constructed and described (Park et al.
3 2005b) (Fig. 1a). The maize CRT (NCBI accession number: AF190454) open reading
4 frame was cloned into the *Sac*I site of pE1775 binary vector (Lee et al. 2007) (Fig. 1a),
5 and the pE1775::CRT and pCaMV::sCAX1 were introduced into *Agrobacterium*
6 *tumefaciens* strain LBA 4404 (Hoekema et al. 1983) using the freeze-thaw method
7 (Holsters et al. 1978). The pE1775 expression vector contains a superpromoter, which
8 consists of a trimer of the octopine synthase transcriptional activating element affixed
9 to the *mannopine synthase2'* (*mas2'*) transcriptional activating element plus minimal
10 promoter, and has been proved to be a strong promoter when being expressed in
11 tobacco and maize (Lee et al. 2007). 35SCaMV promoter was intentionally avoided to
12 drive *CRT* gene because previous studies suggest that two transgenes driven by the
13 same promoter might cause silencing of one or both genes (Park et al. 1996).
14
15 **Plant material, transformation, and growth conditions**
16 Tobacco (*Nicotiana tabacum* L.) cultivar KY14 was used in this study. Tobacco
17 transformation was performed via *Agrobacterium*-mediated leaf disk transformation
18 method as previously described (Horsch et al. 1985). Seeds were surface-sterilized
19 and germinated on MS inorganic salt medium (Murashige and Skoog 1962) with 30 g
20 l^{-1} sucrose, pH 5.7, and solidified using 8 g l^{-1} agar (PhytoTechnology, Shawnee
21 Mission, KS, USA). Transformants were selected on standard medium containing 100
22 μg ml^{-1} kanamycin for *sCAX1*-, 50 μg/mL hygromycin for *CRT*-, and 100 μg ml^{-1}

kanamycin plus 50 μg ml^{-1} hygromycin for *sCAX1*- and *CRT*-co-expressing tobacco. Tobacco plants were grown in a greenhouse as previously described (Hirschi 1999). For ion sensitivity analysis, surface-sterilized seeds were germinated in MS media. Ten days after plating, the seedlings were transferred to MS media supplemented with the appropriate ion. To make media deficient in Ca^{2+}, we removed the $CaCl_2$ from the nutrient solution. The T1 and T2 tobacco plants were grown in the greenhouse under a 16-h photoperiod within a temperature range of 25 °C to 30 °C. Leaves from 2-month-old T2 generation tobacco plants were sampled for Ca^{2+} concentration analysis.

Tomato (*Solanum lycopersicum* 'Rubion') transformation was performed via *Agrobacterium*-mediated transformation method using cotyledon and hypocotyls explants as previously described (Park et al. 2003). *A. tumefaciens* LBA 4404 was used for generating stable transgenic plants. After inoculation with *A. tumefaciens*, the plant cultures were maintained at 25 °C under a 16-h photoperiod. After 6 to 8 weeks, regenerated shoots were transferred to rooting medium for additional six weeks. The temperature of the greenhouse was maintained within a range of 25 °C to 28 °C.

T2 generation of tomato plants were grown in the greenhouse with the same conditions described above. We manually pollinated the flowers and marked the date of pollination. The number of healthy and BER fruits was counted and the BER ratio was examined. The fruits of 40-day after pollination (40 DAP) were harvested for Ca^{2+} content determination.

1 **DNA isolation and DNA gel blot analysis**

2 Genomic DNA of tobacco and tomato was isolated from 100 mg of fresh leaves using
3 the DNeasy Plant Mini-Kit (Qiagen, Valencia, CA, USA) according to the
4 manufacturer's instructions. DNA gel analysis was carried out as described previously
5 (Park et al. 2009). Genomic DNA (5-10 μg) was digested with *Xba*I, separated in a
6 0.9% (w/v) agarose gel by electrophoresis and blotted on to a nylon membrane
7 (Zeta-Probe GT membrane, BioRad Laboratories, Hercules, CA, USA). The probe for
8 the *sCAX1* gene was isolated by digesting pBluscript::sCAX1 (Park et al. 2009). The
9 membranes were pre-hybridized at 65°C in 7% sodium dodecylsulphate (SDS) and
10 0.25 M Na_2HPO_4 for 3 hours, and then hybridized overnight at 65°C in the same
11 solution containing the probe labeled by NEBlot Phototope Kit (New England Biolabs,
12 Beverly, MA, USA). Membranes were washed twice for 40 min each with 20 mM
13 Na_2HPO_4 and 5% SDS at 65 ^{o}C and then washed twice again for 30 min each with 20
14 mM Na_2HPO_4 and 1% SDS at 65 ^{o}C. The signal was detected using the
15 Phototope-Star Detection Kit (New England Biolabs).

16

17 **RNA isolation, RT-PCR, and RNA gel blot analysis**

18 Total RNA of tobacco and tomato was extracted from leaves using RNeasy Plant Mini
19 Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA
20 for RT-PCR was treated with RNase-free DNase prior to the synthesis of first-strand
21 cDNA by oligo (dT) priming using moloney murine leukaemia virus-reverse
22 transciptase (BD Biosciences Clontech, Palo Alto, CA, USA). One microliter of the

1 reverse transcription reaction solution was used as a template in a 25 μl PCR solution.
2 Total RNA (7 μg) was separated on a 1.2% agarose gel containing 1.5% formaldehyde,
3 and blotted on to a Zeta-Probe GT membrane according to the manufacturer's
4 instructions. Hybridization and washing were performed as described previously in
5 DNA gel blot analyses (Park et al. 2009).
6
7 **Ca^{2+} and other mineral analysis**
8 The tobacco leaves and tomato fruits were dried at 70 ^{o}C for 4 d. A total of 0.5 g (dry
9 weight) of fruits was digested for analysis as described (Park et al. 2005a). Calcium
10 content per gram of dry weight was determined by inductively coupled plasma
11 emission spectrophotometry (Spectro, Kleve, Germany).
12
13 ## Results
14
15 **Generation of *sCAX1*-, *CRT*-, and *sCAX1*- and *CRT*-co-expressing tobacco and**
16 **tomato plants**
17 The temporal and spatial regulation of s*CAX1* is crucial for proper modulation of Ca^{2+}
18 with plant cells (Park et al. 2005a). The 35S promoter confers strong constitutive
19 expression in plants, and is often used to give high level expression of a given gene
20 (Benfey et al. 1990). In previous studies, various *sCAX1*-expressing lines under the
21 control of the 35S promoter showed symptoms similar to Ca^{2+} deficiency (Hirschi
22 1999; Park et al. 2005a), and this promoter may therefore be used effectively to

1 identify the capacity to regulate Ca^{2+}-related cellular dysfunction in
2 *sCAX1*-expressing plants through manipulation of CRT. Initially 18 *sCAX1*- and 20
3 *CRT*- expressing lines were generated, respectively, and then we co-transformed *CRT*
4 into two independent *sCAX1*-expressing T2 homozygous tobacco lines (sCAX1-1 and
5 sCAX1-2). The stable integration of the *35S::sCAX1* chimeric construct in the
6 genome of tobacco plants that were used for *CRT* co-transformation was confirmed by
7 Southern-blot analysis (Fig. 1b). The line we termed sCAX1-2 appeared to contain a
8 single-copy insertion, while line sCAX1-1 and sCAX1-5 had more than one
9 integration event (Fig. 1b). Twenty independent *sCAX1*- and *CRT*-co-expressing
10 tobacco lines (hereafter as *sCAX1+CRT*) were generated by *CRT* co-transformation.
11 Expression of *sCAX1* and *CRT* transcripts were measured in T1 transgenic lines by
12 RNA gel blot analysis. Two *sCAX1*- and *CRT*-co-expressing lines sCAX1+CRT-2 and
13 -3 appeared to show stronger bands compared to other lines sCAX1+CRT-13,
14 sCAX1-1, or CRT-1 (Fig 1c). The intensity of the signal in sCAX1+CRT-2 and -3 may
15 result from high-level of expression in those particular lines by transformation
16 variability, various technical issues such as an excess of loaded total RNAs, or the
17 possible co-transformation effect of two different genes. Regardless, the results
18 suggest that *sCAX1* and *CRT* transcripts were expressed only in the sCAX1 and CRT
19 transgenic lines, respectively; while both *sCAX1* and *CRT* transcripts accumulated in
20 the sCAX1+CRT-2, -3, and -13 transgenic lines (Fig 1c).
21 Previous tomato studies demonstrate that *sCAX1* expression also causes apical
22 burning and the development of distinct necrotic lesions in the distal portion of fruits

(BER). Thus, we were interested in determining whether co-expression of *CRT* in *sCAX1*-expressing tomato plants would alleviate the symptoms. Initially 24 *sCAX1*- and 15 *CRT*- expressing lines were generated, respectively, and then we co-transformed *CRT* into a *sCAX1*-expressing - 13 (a single-copy insertion) T2 homozygous tomato line that showed severe Ca^{2+} deficiency-like symptoms including BER (data not shown). Twelve independent *sCAX1+CRT*-expressing tomato lines were generated. Two of each *sCAX1*-2 and 13, *CRT*-9 and 21, and *sCAX1+CRT*-4 and 5 expressing transgenic lines were randomly selected and confirmed by Southern-blot and PCR analysis (Fig. 1d,e).

The stable integration of the *CRT* in the genome was confirmed by Southern-blot (Fig. 1d). We found a background band in every line, including wild-type, which might be caused by the endogenous *CRT* in the tomato genome. The Southern-blot result suggests that the CRT-21, sCAX1+CRT-4, and sCAX1+CRT-5 lines contained a single-copy of CRT, while CRT-9 line contained 3 copies of CRT. The integration of *sCAX1* in the genome was confirmed by PCR using s*CAX1* primers (Fig. 1e, Supplementary Table 1). The expression of *CRT* and *sCAX1* was confirmed by RT-PCR using *CRT* and s*CAX1* primers, respectively (Fig. 1f,g, Supplementary Table 1). All the molecular works were conducted using the T2 generation plants.

CRT suppresses *sCAX1*-induced Ca^{2+} deficiency-like symptoms of tobacco and tomato plants

As shown previously (Hirschi 1999), *sCAX1*-expressing tobacco lines including two

1 independent *sCAX1*-expressing T2 homozygous tobacco lines (sCAX1-1 and
2 sCAX1-2, Fig. 2a, b) that were used for *CRT* co-transformation have altered
3 morphology and growth characteristics. All the *sCAX1*-expressing lines displayed
4 necrosis on the tips of the new leaves from a young stage, which is a Ca^{2+}
5 deficiency-like symptom (Fig. 2c). In addition to the necrosis, all the
6 *sCAX1*-expressing tobacco plants showed severe stunting (Fig. 3a, bottom). In
7 contrast, after introducing the *CRT* into *sCAX1*-expressing tobacco plants, the
8 symptoms were alleviated (Fig. 2d and 3a, top).

9 To establish that the growth phenotypes were due to co-expression of the *CRT*,
10 40-45 each of *sCAX1+CRT*-expressing T2 generation plants from 5 independent lines
11 (sCAX1+CRT-2, -3, -6, -13, and -27) were analyzed to determine if *CRT* segregated
12 with the robust growth phenotype. As shown in Fig. 3b (right) and 3c, 4 of 5 lines
13 showed a segregation pattern of 3:1 for the robust growth phenotype (Supplementary
14 Table 2), and all the *CRT*-co-expressing lines were healthy while the absence of *CRT*
15 caused the reappearance of the symptoms associated with *sCAX1*-expression [Fig. 3b
16 (left) and 3c]. This result suggests that CRT contributes to the recovering of
17 *sCAX1*-expressing tobacco plants with Ca^{2+} deficiency-like symptoms.

18 To determine how the expression of *sCAX1*, *CRT* and *sCAX1+CRT* alters Ca^{2+}
19 concentration in the cells, we measured the total accumulation of Ca^{2+} in the tobacco
20 leaves in T2 generation transgenic plants. As shown in Fig. 3d, *sCAX1*- and
21 *sCAX1+CRT*-expressing tobacco plants accumulated significantly more (up to 25%)
22 Ca^{2+} than wild-type plants; however, *CRT*-expressing tobacco plants did not

1 significantly enhance Ca^{2+} accumulation as compared with wild-type plants. In
2 addition, expression of *sCAX1*, *CRT* or *sCAX1+CRT* did not affect the accumulation
3 of other minerals (Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, Supplementary Fig. 1).
4 In order to ascertain whether CRT can suppress *sCAX1*-induced adverse
5 symptoms in tomato plants, we introduced *CRT* into *sCAX1*-expressing tomato plants.
6 As shown in Fig. 4a and Supplementary Fig. 2, the necrosis in leaf tips caused by
7 *sCAX1*-expressing was alleviated by the co-expression of *CRT*. Furthermore, when we
8 counted the number of the BER and healthy fruits of wild-type, *sCAX1*-, *CRT*-, and
9 *sCAX1+CRT*-expressing T2 generation transgenic plants, respectively, the results
10 showed that the BER ratio could be reduced by introducing *CRT* to the
11 *sCAX1*-expressing plants. Although the ratio of BER in *sCAX1+CRT*-expressing
12 plants was not statistically different from that of *sCAX1*-expressing plants, because
13 the BER ratio shows a large variation among different plants even in the same line,
14 the BER symptom in *sCAX1+CRT*-expressing plants was indeed less severe than that
15 in *sCAX1*-expressing plants according to our day-to-day observation (Fig. 4b, c, and
16 data not shown).
17 To determine how the co-expression of *CRT* in s*CAX1*-expressing tomato alters
18 Ca^{2+} concentration in the fruit cells, the total accumulation of Ca^{2+} in the tomato fruits
19 of wild-type, *sCAX1*-, *CRT*-, and *sCAX1+CRT*-expressing T2 generation plants was
20 analyzed. All the *sCAX1*- and *sCAX1+CRT*-expressing tomatoes showed
21 significantly higher Ca^{2+} content than wild-type tomatoes (Fig. 4d). However, among
22 15 *CRT*- expressing tomato lines, the majority of these lines did not significantly

enhance Ca^{2+} content as compared to wild-type tomatoes while the fruits of line #9 and #21 increased ~ 9% and ~ 40% more Ca^{2+} than wild-type fruits, respectively (Fig. 4d).

CRT suppresses *sCAX1*-induced ion sensitivity in tobacco lines under ion imbalance growth condition

We further tested whether introducing CRT could mitigate the ion sensitivity caused by sCAX1. After *in vitro* growing lines on standard MS media for 14 d, wild-type and transgenic seedlings (sCAX1-1 and -2; CRT-1; sCAX1+CRT-2, -3, -6, -13, and -27) were transferred to media containing various concentrations of Mg^{2+} or K^{+}, or reduced Ca^{2+}. All the *sCAX1*-expressing seedlings were sensitive to the ion imbalance that failed to perturb the growth of wild-type and *sCAX1+CRT*-expressing plants. For example, after being transferred in the Ca^{2+}-depleted media, the *sCAX1*-expressing seedlings could not grow and develop leaves (Fig. 5a). In contrast, the *sCAX1+CRT*-expressing seedlings grew vigorously without any abnormal morphological developments (Fig. 5a). In the medium containing 50 mM $MgCl_2$, the *sCAX1*-expressing seedlings also showed hypersensitivity to the stress, such as necrotic lesions in the young leaves and stunted growth (Fig. 5b); however, the *sCAX1+CRT*-expressing seedlings did not display any adverse growth (Fig. 5b). The sensitivity of *sCAX1*-expressing tobacco to K^{+} salt stress was not as severe as the Ca^{2+} or Mg^{2+} growth phenotypes. However, after transferring the seedlings to the media containing 100 mM KCl for 60 days, the necrotic lesions displayed on the

sCAX1-expressing leaf tips, but not on the leaves of *sCAX1+CRT*-expressing plants (Fig. 5c).

Discussion

Conventional breeding strategies for mineral biofortification of crops rely on germplasm with limited genetic variation for many traits (White and Broadley 2009). In some cases, genetic diversity can be increased by crossing to distant related species and movement of the traits slowly into the agronomically useful cultivars. However, the variation in a trait, in particular Ca^{2+} concentration, may not cover the range desired for agronomic value. Thus, breeders may not have the appropriate level of genetic variation in Ca^{2+} concentration among varieties. Our genetic engineering approach allows over-expression of Ca^{2+} transporter genes and expression to a level not present in germplasm. However, a major impediment for the development of Ca^{2+}-biofortified crops using Ca^{2+} transporters is that the transgenic lines expressing *sCAX1* dramatically increase Ca^{2+} content in their tissues, but also display severe Ca^{2+} deficiency-like symptoms, leading to significant yield losses (Hirschi 1999; Park et al. 2005a). Previous studies in *Arabidopsis* suggest that CRT plays a key role in the regulation of Ca^{2+} status of the plant ER and that the ER, in addition to the vacuole, is an important Ca^{2+} store in plant cells (Persson et al. 2001). In fact, *Arabidopsis* plants over-expressing a version of CRT contained up to 35% more total Ca^{2+}, and the increased Ca^{2+} sequestered by the CRT appeared to benefit plants when grown in a

1 Ca^{2+} deficient situation (Wyatt et al. 2002). Results from these studies also suggest
2 that the CRT-mediated alteration of the ER Ca^{2+} pool could potentially make Ca^{2+}
3 more readily accessible for release into the cytosol and further strengthens the notion
4 that the increased Ca^{2+}-buffering capacity generated by overproduction of CRT helps
5 maintain Ca^{2+} homeostasis.

6 There are at least two different groups of CRT isoforms, CRT1/CRT2 and CRT3,
7 in higher plants (Persson et al. 2003). Different isoforms of CRT exhibit differences in
8 the tissue-specific and stress-dependent expression patterns, indicating that they are
9 involved in different pathways for their functions in plants (Jia et al. 2009). Among
10 different CRT isoforms, CRT1 can substitute for animal CRTs in terms of modulation
11 of Ca^{2+} homeostasis (Christensen et al. 2008). In addition, the role of maize CRT1 in
12 plant responses to stress has been previously studied (Wyatt et al. 2002; Akesson et al.
13 2005). Thus, a maize CRT1 was chosen in this study to further investigate whether
14 co-expression of the *CRT1* may mitigate the Ca^{2+} deficiency-like symptoms caused by
15 expression of *sCAX1*. Indeed, co-expression of a maize *CRT* mitigates the Ca^{2+}
16 deficiency-like symptoms including tip burning and BER (Figs. 2, 3, and 4) and the
17 hypersensitivity to ion imbalance (Fig. 5) caused by expression of *sCAX1* in tobacco
18 and tomato plants. Although *CRT* expression alone was not sufficient to dramatically
19 alter the Ca^{2+} content and incidence of BER in this study, our results here suggest that
20 combining expression of transporters and binding proteins may be a strategy to alter
21 the concentration of Ca^{2+} without negatively impacting plant growth and
22 development.

CRT is also known to harbor chaperone-like functions that may influence protein folding by interacting with unfolded proteins (Crofts and Denecke 1998). Indeed, recent studies indicate that AtCRT1a (also known as AtCRT1) and CRT1b family members are components of a general ER chaperone network and AtCRT1a restores putative folding deficiencies (Christensen et al., 2008; 2010). Furthermore, CRT expression is induced by biotic and abiotic stresses and may ensure plants adapt to various stresses (Jia et al., 2009). Therefore, it cannot be ruled out that co-expression of *CRT* in *sCAX1*-expressing lines could mitigate adverse effects by working as a stress-inducible chaperone and/or a positive regulator in stress responses.

Most mature plant cells have a central vacuole, which often takes up more than 80% of the cell volume (Martinoia et al. 2000). The vacuole is considered to be the largest intracellular storage compartment for Ca^{2+} (Gelli and Blumwald 1993), and fluxes of Ca^{2+} across the vacuole are similar in magnitude to those occurring across the plasma membrane (Bush 1995). The plant ER, like the vacuole, is thought to function as a substantial Ca^{2+} storage compartment (Iwano et al. 2009). In animals, total Ca^{2+} concentration can approach micromolar concentrations in the mammalian sarcoplasmic reticulum (SR) (Zucchi and RoncaTestoni 1997). Measurements of Ca^{2+} efflux from plant ER vesicles indicate that there is rapid exchange of Ca^{2+} across the ER (White and Broadley 2003). Our data suggest that increased expression of Ca^{2+} binding proteins on the ER can ameliorate the adverse effects caused by increasing sequestration of Ca^{2+} into the vacuoles. Recent technological advances should enable future studies to make a detailed analysis of Ca^{2+} dynamics in different cellular

1 compartments to decipher the temporal and spatial characteristics of Ca^{2+} signatures
2 caused by altered *sCAX1* and *CRT* expression (Krebs et al. 2012).

3 In Arabidopsis mutants where CAX activity is greatly reduced, the lines show
4 3-fold more apoplastic Ca^{2+} (Conn et al. 2011). On the other hand, when *sCAX1*
5 expression is increased in tomato plants, apoplastic concentration of Ca^{2+} are reduced
6 (de Freitas et al. 2011). Depleting the apoplastic Ca^{2+} pool by expression of *sCAX1*
7 may cause the Ca^{2+} deficiency-like symptoms. One of the important functions of
8 apoplastic Ca^{2+} is cross-linking the homogalacturonans for the biosynthesis of cell
9 wall (Cosgrove 2005). Thus, reducing the apoplastic Ca^{2+} concentration in
10 *sCAX1*-expressing plants could disrupt the cell wall biosynthesis and further results in
11 growth stunting, tip burning and BER, especially in the tissues that the cell division
12 and wall formation are most rapid (Figs. 2, 3 and 4). Furthermore, recent studies show
13 that suppressing expression of *pectin methylesterases (PMEs)* in tomato fruit reduces
14 the amount of Ca^{2+} bound to the cell wall, subsequently increasing Ca^{2+} available for
15 other cellular functions and, thereby, reducing fruit susceptibility to BER(de Freitas et
16 al. 2012). Therefore, future research may focus on elucidating the effects of
17 co-expression of *CRT* and *sCAX1* on the distribution/partitioning of symplastic and
18 apoplastic Ca^{2+}.

19 Ca^{2+} disorders, likely involving altered CAX activity, may be responsible for
20 losses in crop production (Ho and White 2005). These putative Ca^{2+} disorders have
21 been thought to develop similarly (White and Broadley 2003) and to be associated
22 with a Ca^{2+} deficiency within the cells (Saure 2001). BER in tomato and bitter pit in

1 apples may also be linked to changes in CAX activity (Park et al. 2005a; de Freitas et

2 al. 2010). To explain the primary causes of BER, two hypotheses have been

3 considered, 1) Ca^{2+} deficiency and 2) aberrant Ca^{2+} homeostasis. The majority of

4 studies on BER in recent years have proposed that Ca^{2+} imbalance events at the

5 cellular level, triggered by environmental stresses, may result in aberrant intracellular

6 Ca^{2+} signals, ultimately leading to BER. It is suggested that this phenomenon might

7 be a consequence of aberrant cytosolic Ca^{2+} regulation, and therefore spatial and

8 temporal control of cellular Ca^{2+} concentration is a key factor determining incidence

9 of Ca^{2+}-related physiological disorders (Hirschi 2004; Ho and White 2005; Park et al.

10 2005a; Karley and White 2009; White and Broadley 2009; Dayod et al. 2010; de

11 Freitas et al. 2011). Regardless of mechanisms, our work here shows that elevated

12 expression of *CRT* can reduce the severity of growth abnormalities caused by

13 increased CAX activity.

14 Utilization of the *sCAX1* for Ca^{2+} biofortification have been extensively

15 investigated in various horticultural crop species (carrot, potato, tomato, lettuce) since

16 the expression of *sCAX1* can dramatically improve the Ca^{2+} accumulation in their

17 edible tissues (Hirschi 1999; Park et al. 2004; Park et al. 2005a; Park et al. 2005b;

18 Park et al. 2008; Park et al. 2009). Interestingly, not all the increased Ca^{2+} in the

19 transporter-modified carrots was bioavailable (Morris et al. 2008). This may be due

20 to a fraction of the extra Ca^{2+} being bound to antinutrients within the carrot (Hirschi

21 2009). This serves as a cautionary example for scientists that assume that all

22 increases in nutrient content directly equate to increased bioavailability. However, the

1 modified carrots are a better source of Ca^{2+} because total Ca^{2+} absorbed was higher.
2 Although we postulate that the Ca^{2+} content has increased within the vacuoles of the
3 modified carrots, we have not yet addressed the intracellular Ca^{2+} redistribution in
4 these plants experimentally. We postulate that co-expressing various transporters and
5 *CRTs* will differentially increase total Ca^{2+} content and the fractional absorption of
6 Ca^{2+} in animals. However, feeding studies must be conducted to address the
7 bioavailability issues in the double transformants, including the CRT+sCAX1
8 transformed crops.
9 Our working hypothesis is that the Ca^{2+} content within these double transgenic
10 plants is more evenly distributed throughout the plant cells. However, in order to
11 decode the relationship between expression of transporters and binding proteins and
12 location of Ca^{2+} within the cell, we must determine the spatial resolution of Ca^{2+}
13 within the plant (Punshon et al. 2009; Conn et al. 2011; Punshon et al. 2012). Various
14 techniques exist to visualize the distribution and abundance of elements within plants.
15 These techniques are useful because, in contrast with bulk or volume-averaged
16 measures (such as inductively coupled plasma mass spectroscopy, ICP-MS) where the
17 sample is homogenized, the confinement of elements within specific plant organs,
18 tissues, cells and even organelles can be seen (Punshon et al. 2012). The potential of
19 synchrotron x-rays in spatially resolved elemental imaging in plants has begun to be
20 realized (Punshon et al. 2009). In fact, this work has recently been done to
21 demonstrate the alterations of Ca^{2+} partitioning in seeds of Arabidopsis lines altered in
22 CAX expression (Punshon et al. 2012), it will certainly be interesting to apply this

1 technology to the edible portions of crops co-expressing both *sCAX1* and *CRT*.

2 In conclusion, while genetic engineering strategies to increase Ca^{2+} content by
3 expression of a single gene (either *sCAX1* or *CRT*) alone have provided promising
4 results, co-expressing of *CRT* and *sCAX1* enhances the Ca^{2+} content of plants without
5 any apparent detrimental effects potentially caused by sCAX1 expression.
6 Manipulation of the partitioning of nutrients across various endomembranes may be a
7 means to increase plant nutrient content while maintaining crop productivity.
8

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10
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17

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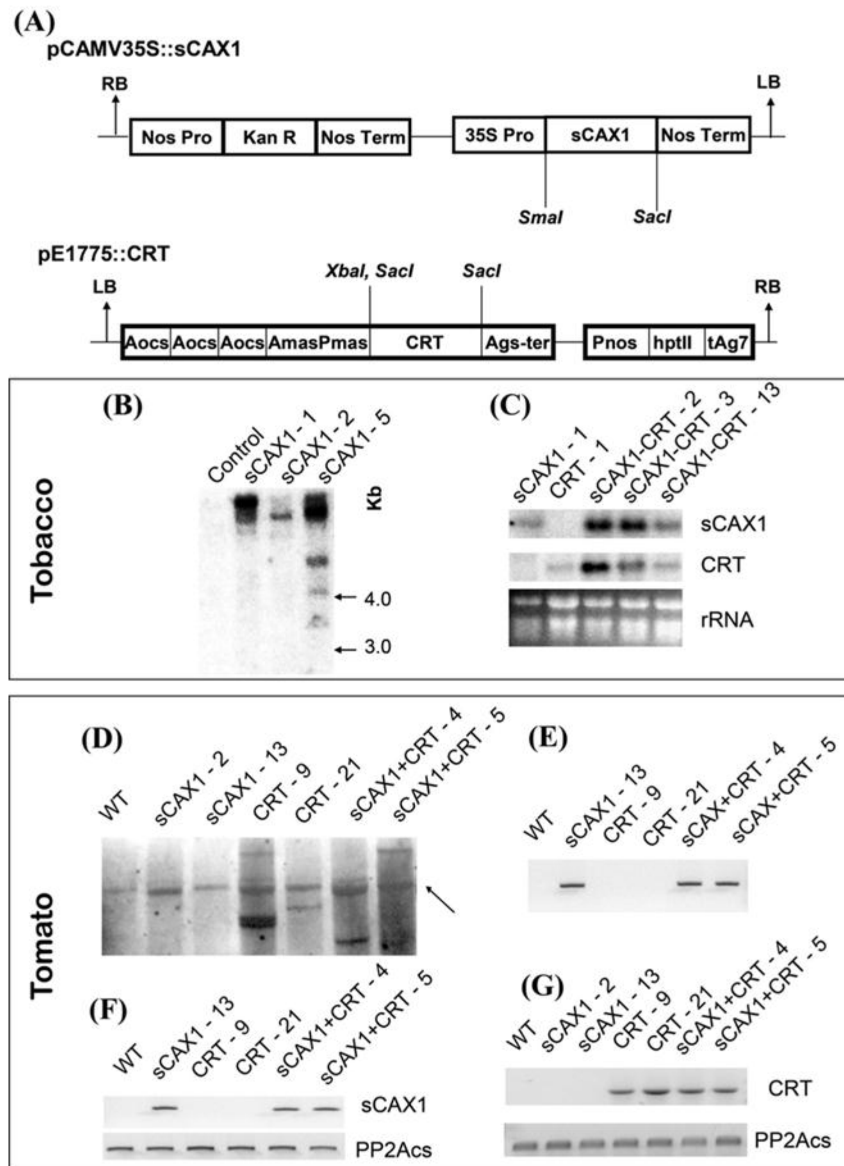
1 # Figures



2

3 **Fig. 1** Molecular analyses of *sCAX1*-, *CRT*- and *sCAX1+CRT*-expressing tobacco and

4 tomato plants. (a) T-DNA regions of pCaMV35S::sCAX1 and pE1775::CRT. RB,

5 Right border; LB, left border; Nos-pro, nopaline synthase promoter, Kan R, the gene

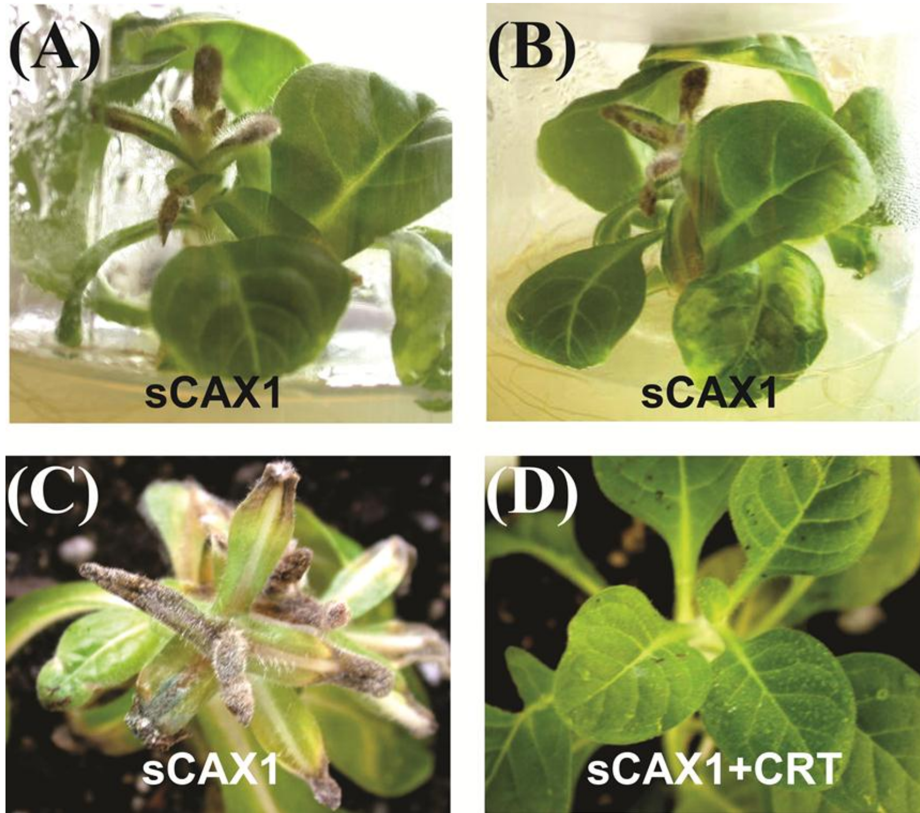
6 conferring resistance to kanamycin, neomycin phosphotransferase (NPTII); Nos-ter,

7 nopaline synthase terminator. 35S pro, CaMV 35S promoter; sCAX1, short cut cation

8 exchanger 1 coding region; Aos, octopine synthase transcriptional activating element;

9 AmasPmas, mannopine synthase 2' activating and promoter elements; CRT, maize

calreticulin coding region; ags-ter, polyA addition signal from the agropine synthase gene. hptII, gene conferring resistance to hygromycin; Pnos, mopaline synthase promoter; tAg7, poly A addition signal for T-DNA gene 7. (b) Southern-blot analysis of transgenic tobacco plants. Ten micrograms of tobacco genomic DNA were digested with *SacI*, and hybridized with the *sCAX1* probe. (c) Northern-blot analysis of transgenic tobacco plants. Ten micrograms of total RNA from leaves were hybridized with *sCAX1* and *CRT* probe, respectively. Ethidium bromide-strained rRNA (bottom) is shown as a loading control. (d) Southern-blot analysis of transgenic tomato plants with CRT probe. Ten micrograms of tomato genomic DNA were digested with *XbaI*, and hybridized with *CRT* probe. The arrow indicates the endogenous tomato *CRT* gene that was detected by maize *CRT* probe. (e) PCR detection of *sCAX1* in genomic level. (f) RT-PCR detection of the expression of *sCAX1*. (g) RT-PCR detection of the expression of *CRT*. *SlPP2Acs* was used as tomato housekeeping gene.



1

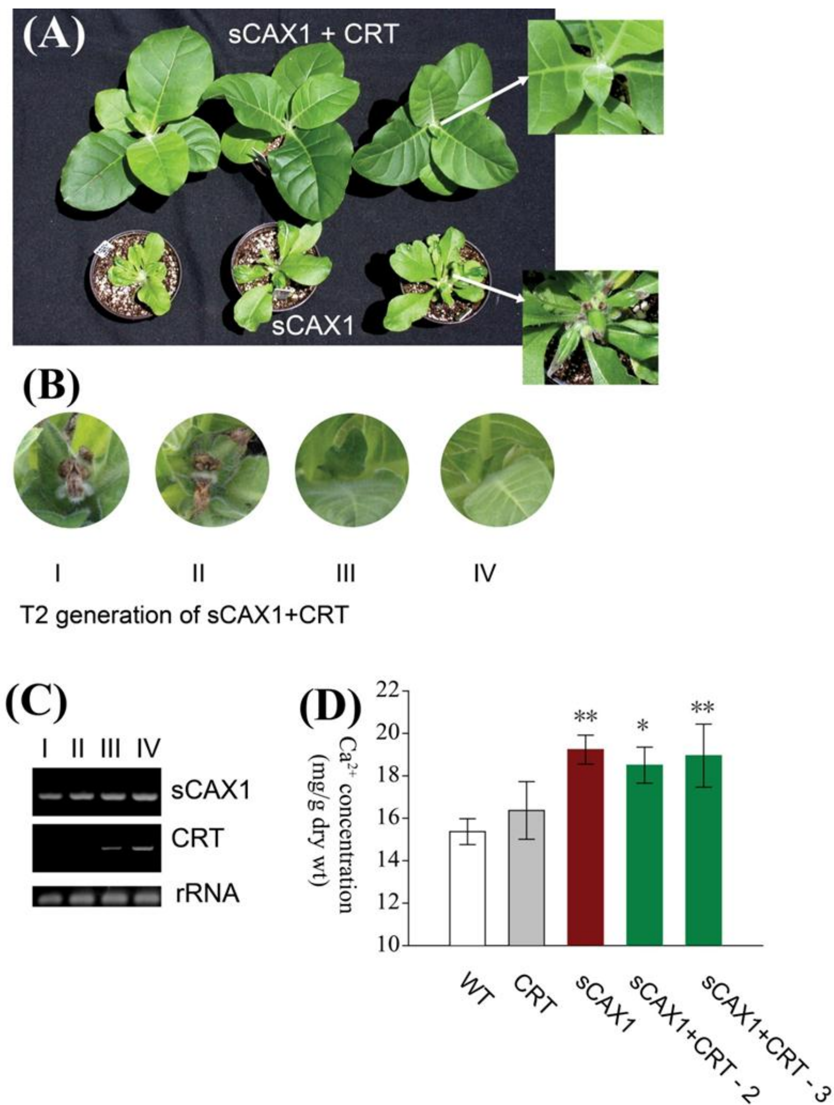
2 **Fig. 2** Morphology of *sCAX1*-, and *sCAX1+CRT*-expressing tobacco plants at young

3 stage. (a-b) the *sCAX1*-expressing tobacco plants used for *CRT* transformation. (c) the

4 morphology of *sCAX1*-expressing tobacco seedlings. (d) the morphology of

5 *sCAX1+CRT*-expressing tobacco seedlings.

6



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2 **Fig. 3** Segregation of the Ca^{2+}deficiency-like symptoms. (a) Morphology of T1

3 generation of *sCAX1*-, and *sCAX1+CRT*-expressing tobacco plants. (b) Segregation of

4 the morphology in T2 generation of *sCAX1+CRT*-expressing plants. Some of the

5 plants maintained the normal morphology, but some returned to the

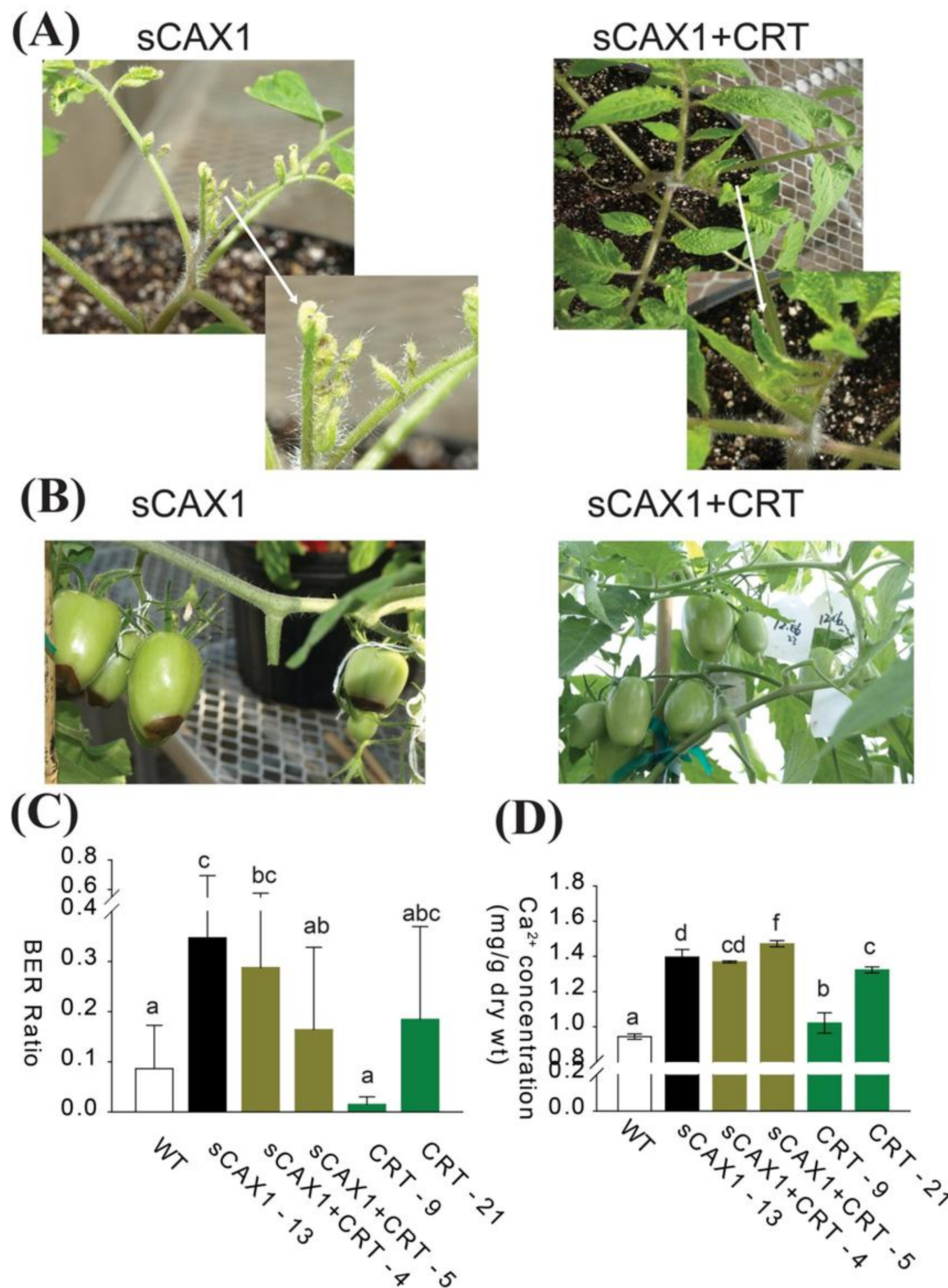
6 Ca^{2+}deficiency-like symptoms. (c) Detection of the expression of *sCAX1* and *CRT* in

7 T2 generation *sCAX1+CRT*-expressing plants by RT-PCR. (d) Ca^{2+} concentration of

8 T2 generation tobacco leaves of different lines. All results shown here are the means

9 of 3 biological replicates, and the error bars indicate the standard deviations (S.D. n=3)

10 (Student t test, * $p<0.05$, ** $p<0.01$).



1

2 **Fig. 4** Expression of *CRT* mitigated the Ca^{2+} deficiency-like symptoms of

3 *sCAX1*-expressing tomato plants. (a) Expression of CRT mitigated the leaf tip burning

4 of *sCAX1*-expressing tomato plants. (b) Expression of *CRT* reduced the BER

5 incidence of *sCAX1*-expressing tomato plants. Left panel, *sCAX1*-expressing tomato

6 plants; right panel, *sCAX1+CRT*-expressing tomato plants. (c) BER ratio of wild-type,

7 *sCAX1*-, *CRT*-, and *sCAX1+CRT*-expressing tomato plants. (d) Concentrations of

1 Ca^{2+} in fruits of wild type, *sCAX1*-, and *sCAX1+CRT*-expressing tomato plants. All

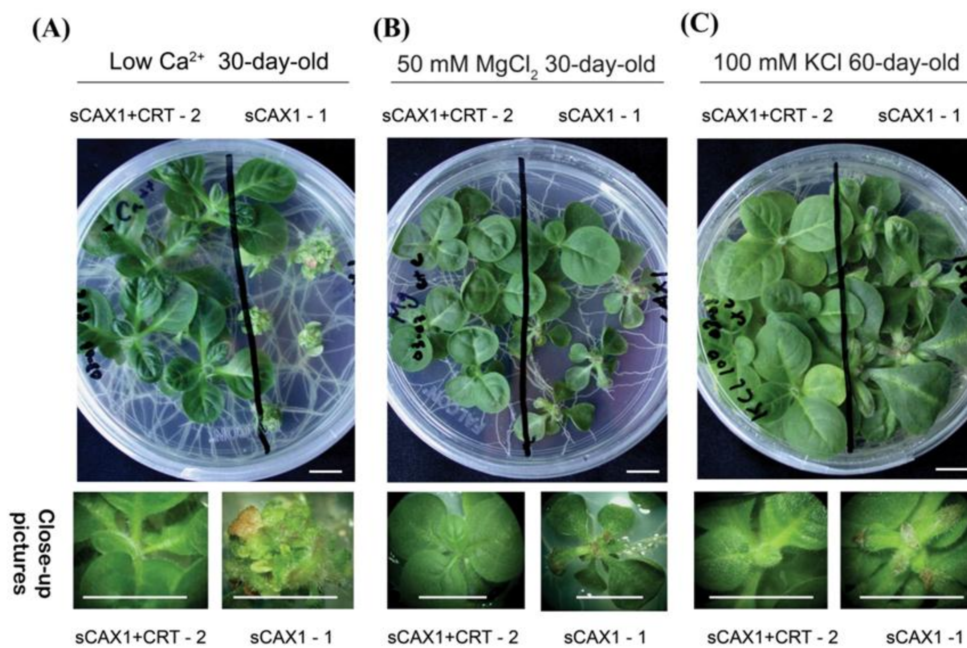
2 results shown here are the means of 3 biological replicates, and the error bars indicate

3 the standard deviations (S.D. n=3). Means accompanied by the same letter are not

4 significantly different using ANOVA analysis ($p<0.05$).

5

6



7

8 **Fig. 5** CRT suppresses *sCAX1*-induced ion sensitivity in tobacco plants. (a) Tobacco

9 seedlings grown in medium with low Ca^{2+} for 30 days. (b) Tobacco seedlings grown

10 in medium with 100 mM $MgCl_2$ for 30 days. (c) Tobacco seedlings grown in medium

11 with 100 mM KCl for 60 days. Upper panel, overview of the plates; lower panel,

12 close up pictures of *sCAX1+CRT*- (lower left) and *sCAX1*-expressing (lower right)

13 seedlings. Four biological replicates were performed. Scale bar = 1 cm.

14