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ORIGINAL ARTICLE

Modest calcium increase in tomatoes expressing a variant of *Arabidopsis* cation/H^+ antiporter

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Abstract The over-expression of *Arabidopsis CAX1* and *CAX2* causes transgenic tomato plants to reveal severe Ca^{2+} deficiency-like symptoms such as tip-burn and/or blossom end rot, despite there being sufficient Ca^{2+} in each plant part. To correct the symptoms and to moderately enhance the calcium level, a worldwide vegetable tomato was genetically engineered using a modified *Arabidopsis* cation/H^+ antiporter sCAX2A, a mutant form of *Arabidopsis* CAX2. Compared with the wild-type, the s*CAX2A*-expressing tomato plants demonstrated elevated Ca^{2+} levels in the fruits with almost no changes in the levels of Mn^{2+}, Cu^{2+}, and Fe^{2+}. Moreover, expression of s*CAX2A* in tomato plants did not show any significant alterations in their morphological phenotypes. Unlike *35S*::*sCAX1* construct, s*CAX2A* antiporter gene driven by *35S* promoter can be a valuable tool for enriching Ca^{2+} contents in the tomato fruit without additional accumulation of the undesirable cations.

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Introduction

Tomato (*Solanum lycopersicum* L., formerly *Lycopersicon esculentum* Mill.) is an important agricultural commodity and is widely consumed worldwide; however, most tomatoes contain little calcium (Ca^{2+}). Uptake and translocation of cationic nutrients including Ca^{2+} in plants plays an essential role in physiological processes such as signal transduction, growth, and development (Flowers and Colmer 2008; Wang et al. 2006). Among them, Ca^{2+} plays a fundamental role in plant membrane stability, cell wall stabilization, and cell integrity (Hirschi 2004). Two *Arabidopsis* Ca^{2+}/H^+ antiporter genes, *CAX1* and *CAX2*, can function in yeast (*Saccharomyces cerevisiae*) mutants as vacuolar Ca^{2+} transporters (Hirschi et al. 1996). The transport properties of CAX2 suggested the potential for broad substrate specificity including Ca^{2+} and Mn^{2+}, whereas CAX1 appeared to be a specialized Ca^{2+} transporter. Subsequent studies have demonstrated that CAX2 can also transport metallic ions such as Cd^{2+} and Zn^{2+} (Hirschi et al. 2000; Pittman et al. 2004; Schaaf et al. 2002; Shigaki et al. 2003). To identify the domains that determine Mn^{2+} specificity in CAX2, six CAX2 variants without the N-terminal autoinhibitory domain (i.e., without the first 42 amino acids) (sCAX2s) were constructed by replacing the regions of divergent sequence in CAX2 with the corresponding regions from CAX1 (Shigaki et al. 2003). When the s*CAX2* variant genes were expressed in yeast mutants defective in vacuolar Ca^{2+} transport, the yeast strains expressing s*CAX2A*

and s*CAX2B* exhibited diminished growth on the Ca^{2+}-containing media, and completely failed to suppress Mn^{2+} sensitivity (Shigaki et al. 2003). These results imply that, among the six s*CAX2* variants, s*CAX2A* and s*CAX2B* are preferred candidates to genetically engineer a robust crop for its moderate but more specific ability of Ca^{2+} transport and diminished ability to accumulate metals such as Mn^{2+}. Indeed, potato tubers expressing s*CAX2B* contained 50–65% more Ca^{2+} than wild-type tubers although these plants are as vigorous as controls, and did not show any significant increase of Mn^{2+} (Kim et al. 2006). In contrast, the transgenic crops ectopically expressing s*CAX1* displayed alterations in plant development and morphology, including increased incidence of blossom-end rot (BER) and tip-burn symptoms despite the elevated Ca^{2+} levels (Hirschi 1999; Park et al. 2004, 2005). Our primary focus here was to evaluate the potential for increasing the Ca^{2+} levels of tomato fruits through expression of a variant cation/H^+ antiporter sCAX2A which has not been characterized on a mode-of-action in plant, and to analyze its potential impact on plant growth and development. This study also suggests that modulation of CAX transporters could make an important contribution toward increasing the value of various agriculturally important crops.

Materials and methods

Tomato genotype, in vitro sowing, and explant preparation

The wild-type tomato in this study was *Solanum lycopersicum* cv. Ailsa Craig (AC) that is a BER-susceptible cultivar. The seeds were surface-sterilized in 70% (v/v) ethyl alcohol for 1 min followed by 1% (v/v) NaOCl for 20 min. The seeds were rinsed three times with sterile distilled water, and then moisturized for 1 h. Twenty seeds were sown in 7 × 10-cm glass vessels containing 25 mL of the sowing medium [MS (Murashige and Skoog 1962) salt, 100 mg L^{-1} myo-inositol, 2 mg L^{-1} thiamine HCl, 0.5 mg L^{-1} pyridoxine HCl and 0.5 mg L^{-1} nicotinic acid] solidified with 8 g/L plant agar (Duchefa Biochemie, Haarlem, The Netherlands) and 10 g L^{-1} sucrose (Duchefa Biochemie). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 18 psi for 20 min. The cultures were incubated at 25°C with illumination for 16 h each day (50 μmol m^{-2} s^{-1}). The cotyledons were detached from the seedlings at the stage of first leaf emerging. For the preparation of explants, 0.5 cm of both distal and basal parts were cut off.

Pre-culture of cotyledon explants on the feeding layer

The NT1 calli of tobacco (*Nicotiana tabacum*) were suspended and cultured in the liquid KCMS medium (MS salt fortified by 200 mg L^{-1} KH_2PO_4, 100 mg L^{-1} myoinositol, 1.3 mg L^{-1} thiamine HCl, 0.2 mg L^{-1} 2,4-D, 0.1 mg L^{-1} kinetin and 30 g L^{-1} sucrose; pH 5.5). Before 1 day for pre-culture, the NT1 calli were spread on the solid KCMS medium followed by covering with a sterile filter paper, and then cultured in the dark. The tomato cotyledon explants were pre-cultured on the filter paper for 16 h under 25°C with illumination (for 16 h each day) (50 μmol m^{-2} s^{-1}).

Bacterial strain and plasmid

The coding region of *sCAX2A* (Shigaki et al. 2003) was cloned into pBI121, which contains the T-DNA cassette consisting of *Nos*-pro/*NptII*/*Nos*-ter/*35S*-pro/*sCAX2A*/*Nos*-ter (Kim et al. 2006). The plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404 using the freeze–thaw method (Holsters et al. 1978), which was subsequently used for the genetic transformation.

Preparation of bacterial inoculum and cocultivation with cotyledon explants

Fifty mL of bacterial culture (OD_{600} = 0.6) were centrifuged at 3,179*g* for 10 min (4°C). After removal of the supernatant, the pellet was suspended in the same volume of MSO (MS salt, 100 mg L^{-1} myoinositol, 2 mg L^{-1} thiamine HCl and 10 g L^{-1} sucrose; pH 5.8), and this suspension was used as the inoculum for infection. The cotyledon explants were immersed in the bacterial inoculum for 10 min and then blotted on a filter paper. After inoculation, they were cultured on the same pre-culture medium for 48 h under 19°C in the dark.

Plant regeneration and acclimatization

Cotyledon explants that had been co-cultivated with the bacteria on the MSO medium were transplanted on a shoot regeneration medium (MS salt, 100 mg L^{-1} myo-inositol, Nitsch vitamins, 2 mg L^{-1} zeatin, 20 g L^{-1} sucrose and 5.2 g L^{-1} Agargel; pH 6.0) with 100 mg L^{-1} kanamycin and 300 mg L^{-1} Timentin. The explants were sub-cultured on the same fresh medium except for the reduced concentration of zeatin (1 mg L^{-1}) every 2 weeks. The regenerated shoots at over 2 cm length were isolated, and transplanted on a rooting medium (MS salt, 100 mg L^{-1} myoinositol, Nitsch vitamins, 20 g L^{-1} sucrose and 5.2 g L^{-1} Difco bacto agar; pH 6.0) with 100 mg L^{-1} kanamycin. The rooted plantlets were

transferred to plastic pots (15 cm in diameter) filled with the compost (vermiculite:perlite 1:1), and then put into a growth chamber for 7 days. The container was covered with polyethylene bags that were opened at increased intervals in order to help the plantlets adapt to the lower humidity of the greenhouse.

Nucleic acid analyses of transgenic tomato

Genomic DNA was extracted from the fresh leaves of tomato plants using a micro-prep isolation protocol modified from Fulton et al. (1995). Seven leaves were put into a 2-mL screw-cap tube and kept on ice. Samples were homogenized in 290 µL of extraction buffer (0.35 M sorbitol, 0.1 M Tris–base, 5 mM EDTA, pH 7.5, and containing 4 mg/mL sodium bisulfate) in a Savant FP120 Fast Prep machine. 290 µL of nuclear lysis buffer [0.2 M Tris–HCl, pH 8, 0.05 M EDTA, pH 8, 2 M NaCl, 2% (w/v) hexadecyltrimethyl-ammonium bromide] and 140 µL 5% sodium lauryl sarcosine were added. The tubes were vortexed and incubated for 40 nm at 65°C. 700 µL of chloroform/octanol (24:1) was added. The samples were vortexed and centrifuged at 8,000*g* for 15 min. The supernatant was transferred to a 1.5 mL tube and 540 µL of cold isopropanol was added for DNA precipitation. DNA was pelleted by micro-centrifugation for 10 min at 13,000*g*. The pellet was washed in 70% ethanol, air-dried, suspended in TE buffer (pH 8.0), and then used for DNA analysis. For PCR amplification of *sCAX2A* gene, one set of primers (5′-GAATGTGAC AGAGCTGATCA-3′ and 5′-GATCGAGGACCCAAT AGCCA-3′) was used. For Southern blot analysis on the PCR-positive plants, the genomic DNA (5–10 µg) was digested with *Xba*I (which made only one cut within the T-DNA), separated by electrophoresis in a 0.9% agarose gel, and then blotted onto Hybond N^+ membranes (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Following the transfer, the membranes were baked at 80°C for 2 h to fix the DNA to the membrane. The probe for the s*CAX2A* gene was isolated from an *Xba*I–*Sac*I (1.2 kb) restriction fragment from the plasmid (Kim et al. 2006). The membranes were pre-hybridized for 3 h using the membrane supplier's protocol and hybridized at 65°C to ^{32}P-labeled random primed DNA probes, synthesized as described by Feinberg and Vogelstein (1983). Following hybridizations for at least 16 h, the filters were washed in 2× SSC, 0.1% SDS, and then in 1× SSC, 0.1% SDS at 65°C. Signal intensity was visualized by autoradiography using Kodak X-OMAT-AR film with two intensifying screens at −80°C. Young stages of tomato fruit (3 cm) were harvested, immediately frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted from the pericarp of at least three individual fruits and analyzed. The RNA was quantified using a spectrophotometer, and 20 µg of the total RNA was fractionated on 1% (w/v) agarose gels containing 7.5% (v/v) formaldehyde and blotted onto Hybond N membrane (Amersham Biosciences) according to the manufacturer's instructions. Following the transfer, the membranes were baked at 80°C for 2 h. Hybridization of membrane was performed as described by Southern hybridization above.

Cation analysis

Ca^{2+} and mineral analysis in each fruit was performed The fruits were dried at 70°C for 4 days, and a total of 0.25 g (dry weight) of fruits was digested using a ternary solution (HNO_3:H_2SO_4:$HClO_4$ 10:1:4 ratio by volume) (Lee et al. 2002). Cation contents per gram of dry weight were determined by inductively coupled plasma emission spectrophotometry (Integra XM2; GBC Scientific Equipment, Australia). One replicate was defined as the average value from three samples.

Results

Generation of *sCAX2A*-expressing tomato lines

Within 8 weeks after transferring of explants into the shoot regeneration medium, most cotyledon explants showed adventitious shoot formation with 3.5–6.1 shoots per explants (Fig. 1a). Collectively, a total of 250 cotyledon explants co-cultivated with *Agrobacterium* were transferred into the selection medium with 100 mg/L kanamycin. Randomly selected 60 shoots with vigorous expanded leaves from 200 cotyledon explants were transplanted on the rooting medium. Out of the 58 shoots that elongated and rooted (Fig. 1b, c), 29 rooted plantlets were successfully acclimatized in a controlled growth room (Fig. 1d).

Genomic DNA was extracted from the leaf tissues of the acclimated plants. Based on the PCR analysis for s*CAX2A*, the transformation frequency of tomato was 96.6% (data not shown). The transformants were also verified for the copy number of the transgene by Southern blot analysis. PCR-positive plants except one plant (plant A23) contained various copies of T-DNA ranging from 1 to 11 (Fig. 2).

We randomly selected five T_0 plants termed A6, A9, A25, A26, and A32 to generate the T_3 homozygous progenies along with T_1 and T_2 generations, which contain only single copy of the construct. By using PCR analysis on T_2 individuals, we selected non-segregating five T_2 populations in which all the plants normally had the integrated T-DNAs (data not shown). As demonstrated in Fig. 3, RNA gel blot also showed that the s*CAX2A* transcripts

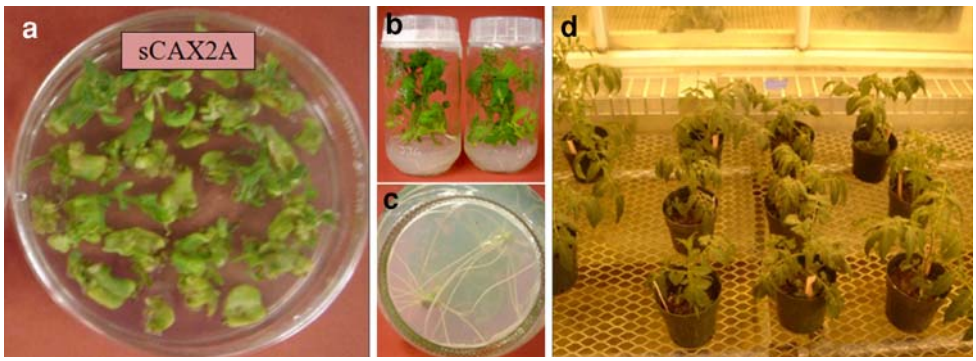

Fig. 1 Regeneration of shoots from cotyledon explants of tomato. **a** Regeneration of adventitious shoots. **b**, **c** Rooted plantlets on a modified MS medium without plant growth regulators. **d** Acclimatized plants in the greenhouse

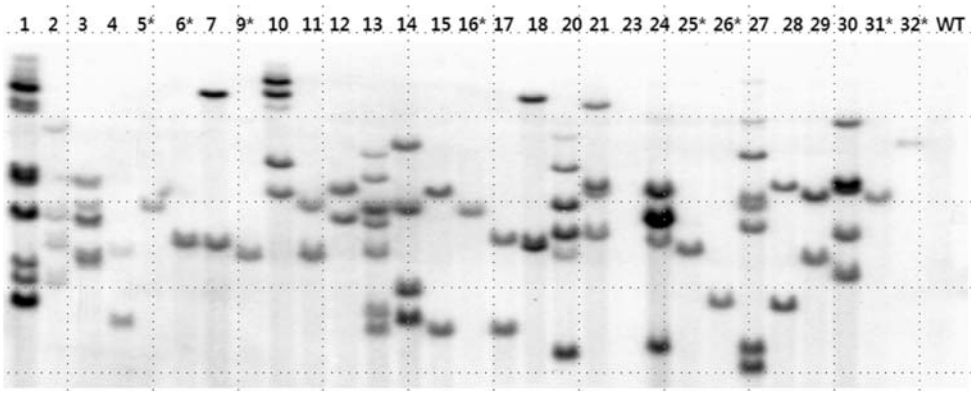

Fig. 2 Southern blot analysis of genomic DNA from PCR-positive tomato plants. The genomic DNA was digested with *Xba*I. *Asterisks* indicate transgenic T_0 plants containing one copy of the construct

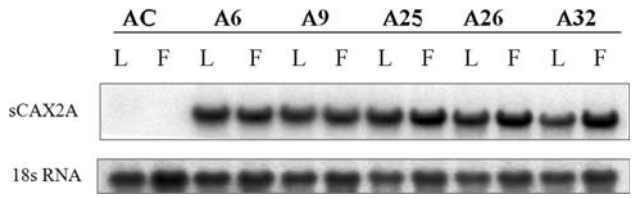

Fig. 3 Northern blot analysis of transgenic T_2 tomato plants. A total RNA was extracted from young leaves (*L*) and green fruit and 20 μg of the total RNA was subjected to RNA gel-blot analysis and hybridized to a ^{32}P-labeled s*CAX2A* gene probe. Rehybridization of 18S rRNA with the same filter shows similar loading of mRNA between samples

equivalently accumulated in young leaves and fruits of the tested transgenic T_2 plants.

Ionic alteration of *sCAX2A*-expressing tomato fruits

To ascertain whether the s*CAX2A* expression altered ion levels, cations including Ca^{2+} were measured in the fruits of T_3 tomatoes. The fruits of *sCAX2A*-expressing plants showed an 45.2–66.7% increase in Ca^{2+} levels depending on fruit developmental stage (breaker stage and 7 days after breaker stage) and transgenic events (A25 and A32), while there were no differences in the Ca^{2+} level of 1 cm fruit as well as other metal ion (Mn^{2+}, Cu^{2+}, and Fe^{2+}) levels in all stages tested (Fig. 4).

Morphological phenotypes of s*CAX2A*-expressing tomatoes

While the s*CAX1*-expressing lines were sensitive to Ca^{2+} deficiency and showed Ca^{2+} deficiency-like symptoms such as tip-burn and BER that were suppressed by addition of Ca^{2+} (Park et al. 2005), the s*CAX2A*-expressing lines were not sensitive to Ca^{2+} deficiency and did not require any additional Ca^{2+} supplementation for normal growth. In addition, both s*CAX2A*-expressing lines and the wild-type control plants grew similarly. In A25 and A32 lines, *sCAX2A* expression did not perturb the morphology, growth, or fruit set (Fig. 5). Moreover, all *sCAX2A* transformants were capable of making viable seed, and the total fruit yield of *sCAX2A* plants was indistinguishable from the wild-type control (data not shown). As the expression of *sCAX1* caused a significant increase in BER occurrence in the supposed BER-tolerant tomato varieties (Park et al. 2005), we chose to express *sCAX2A* in a BER-susceptible tomato line (doubled haploid line 'AC') in order to examine the effect of *sCAX2A* expression on the BER induction. The incidence of BER was equivalent (not

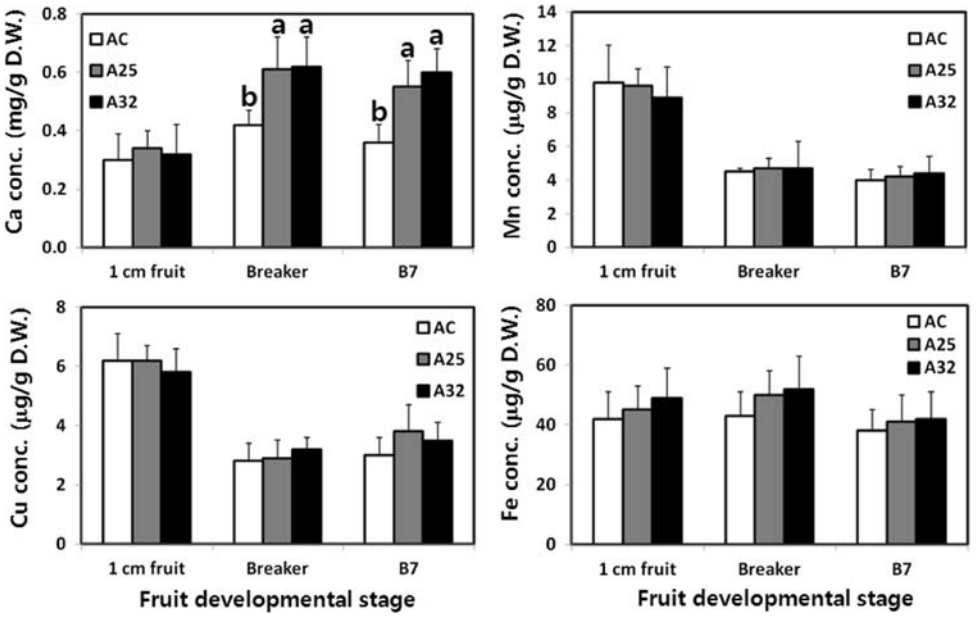

Fig. 4 Cations contents in the fruits of transgenic T_3 events. The contents of cations were measured by inductively coupled plasma emission spectrophotometer. Data represent the mean values (±SE) obtained from three fruits of independent plants. Values in Ca content frame followed by *different letters* are significantly different at the 5% level (Duncan's multiple range test)

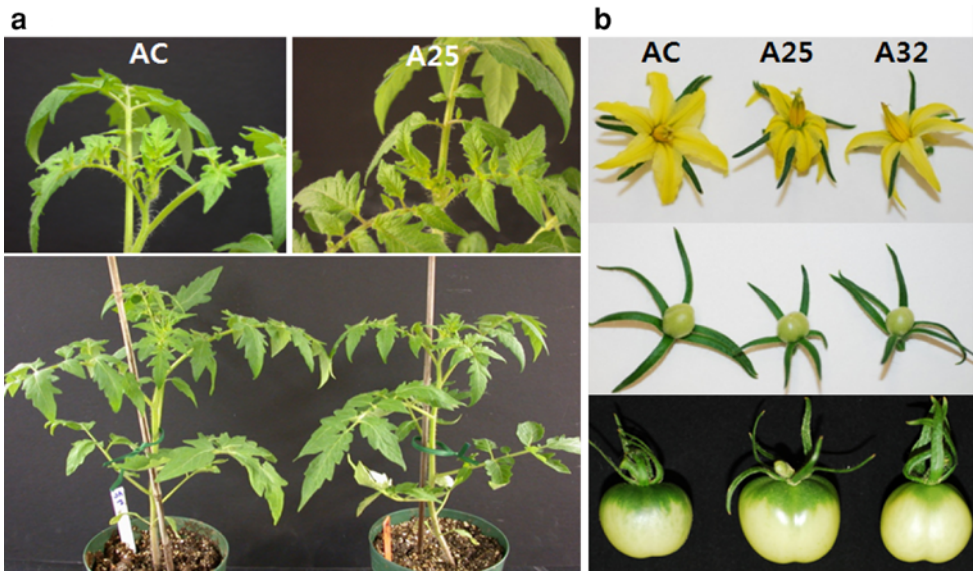

Fig. 5 Phenotypes of s*CAX2A* and wild-type tomato plants. **a** Growth of wild-type control plant (*left AC*) and the s*CAX2A*-expressing plant (*right A25*) after 4 weeks in soil. **b** Flowers, sepals, and young fruits of wild-type (*left AC*) and transgenic lines (*middle A25* and *right A32*). The incidences of tip-burn and BER were not observed in either wild-type or transgenic plants

observed in both lines) in the s*CAX2A*-expressing lines and wild-type control line.

Discussion

We expressed s*CAX2A* in tomato, one of the worldwide fruit vegetables, as a means to increase Ca^{2+} content. Ca^{2+} deficiency symptoms such as tip-burn and BER are the most serious nutritional problem affecting tomato production. A lack of Ca^{2+}, water, and ionic balance can cause BER in Solanaceae crops (Ho and White 2005). Here, we have demonstrated the ability to increase Ca^{2+} levels in tomatoes, which may be a means to deliver more Ca^{2+} to human. Previous studies have demonstrated that expression of Ca^{2+}-signaling components, such as a Ca^{2+} transporter or a Ca^{2+}-binding protein, can be used to increase Ca^{2+} levels in various plants (Wyatt et al. 2002; Hirschi 2004). In this study, we have expressed sCAX2A, the N-terminal autoinhibitory domain-truncated variant with 'A' domain of CAX1 (Shigaki et al. 2003), driven by the cauliflower mosaic virus *35S* promoter. The *35S*-driven s*CAX2A* expression was found to be constitutively expressed in all tomato tissues analyzed, suggesting that sCAX2A cation/H^+ antiport activity would be equally enhanced in all tissues compared to control plants. The *35S*::s*CAX1*-expressing tomatoes displayed the symptoms of Ca^{2+} deficiencies in the soil without additional Ca^{2+} supplementation in the

soil, particularly apical burning and BER (Park et al. 2005). The alterations in plant size, leaf morphology, fruit set, and ripening further emphasize the importance of regulated Ca^{2+} transport in the plant growth and development. Several of these phenotypes, particularly the apical burning, are similar to those of s*CAX1*-expressing tobacco plants (Hirschi 1999). Thus, despite the increased Ca^{2+} in both tobacco and tomato plants, they are suffering from the Ca^{2+} deficiency symptoms. Ca^{2+} supplementation in the soil was required to obtain maximal growth of the s*CAX1*-expressing tomato plants (Park et al. 2005). Some cation/H^+ antiporters are known to have a role in regulating cytoplasmic or vacuolar pH (Yamaguchi et al. 2001). We have not analyzed the effect of *CAX* expression on pH levels in the tomato, and it will be interesting to see whether any morphological changes to the plants are due to altered pH homeostasis. Evidence for Ca^{2+} deficiency as the primary cause of BER has been derived from observations that the blossom end has the lowest content of Ca^{2+} within tomato fruits (Adams and Ho 1993; Nonami et al. 1995). BER is generally associated with the disintegration and increased ion permeability of cells, resulting in loss of turgor and cell fluids invading the intercellular air space, thus causing the watery appearance in the early stages (Shear 1975; Simon 1978). However, despite the increased Ca^{2+} in tomato fruits, the increased incidence of BER in s*CAX1*-expressing tomatoes is perplexing. While the s*CAX1*-expressing tomatoes have increased incidence of BER, no significant difference was observed with the s*CAX2A*-expressing 'AC' line (a doubled haploid line susceptible to BER) when compared to the wild-type (Fig. 5). This may be due in part to the more modest increase in the tomato fruit Ca^{2+} levels seen with s*CAX1* lines. Moreover, s*CAX2A* expression did not perturb the morphology, growth, or fruit set (Fig. 5). The deleterious changes in plant growth caused by *35S*::*sCAX1* expression in tomato plants suggest that further modulation of the expression of Ca^{2+}/H^+ transporters is needed. Here, we have used modified CAX2 (sCAX2A) with A domain of CAX1 and without the N-terminal autoinhibitory domain. The lack of deleterious morphological phenotypes in the *sCAX2A*-expressing plants may correlate with the absence in accumulation of various transition metals. Even at micro-molar concentrations, some transition metals can be particularly toxic (Marschner 1995); therefore, some of the phenotypes in the sCAX1-expressing plants could also be due in part to toxicity induced by Fe^{2+}, Zn^{2+}, or Mn^{2+} accumulation. It is also worth noting that increased Ca^{2+}/H^+ transport activity may activate other proton-mediated transporters (Cheng et al. 2005) to cause alterations in plant growth. Currently, most people obtain their dietary Ca^{2+} from milk-related products; fruits like tomatoes do not contribute significantly to Ca^{2+} intake (Fleming and Heimback 1994). In some areas, communities obtain a majority of their total dietary Ca^{2+} from the consumption of fruits and vegetables (Weaver et al. 1999). Increasing the endogenous levels of Ca^{2+} in commonly consumed fruits should help yield improved dietary Ca^{2+} intakes within many population groups.

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