

Expression of *Arabidopsis CAX4* in tomato fruits increases calcium level with no accumulation of other metallic cations

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ABSTRACT We generated transgenic tomato plants with *Arabidopsis thaliana* H⁺/cation exchanger gene (*CAX4*) by *Agrobacterium*-mediated transformation. We confirmed transgene copy number and transcription by Southern and Northern blot analyses. The intact *CAX4*-expressing tomato (*Lycopersicon esculentum*) fruits contained 63-71% more calcium (Ca²⁺) than wild-type fruits. Moreover, ectopic expression of *CAX4* in tomato fruits did not show any significant increase of the four kinds of metallic cations analyzed (Mg²⁺, Fe²⁺, Mn²⁺, and Cu²⁺). The *CAX4*-expressing tomato plants including their fruits did not show any morphological alternations during whole growth period. These results suggest the enhanced Ca-substrate specificity of *CAX4* exchanger in tomato. Therefore, intact *CAX4* exchanger can be a useful tool for Ca²⁺ nutrient enrichment of tomato fruits with reduced accumulation of undesirable cations.

Introduction

Tomato (*Lycopersicon esculentum*) is an important agricultural commodity. It is widely consumed worldwide even though most tomatoes contain little calcium (Ca²⁺). Calcium plays a fundamental role in plant membrane stability, cell wall stabilization, and cell integrity (Hirschi 2004). Reduced Ca²⁺ in edible plant tissues such as fruit negatively impacts total yield. Plant tissues with low [Ca²⁺] are more susceptible than tissues with normal Ca²⁺ levels to some parasitic diseases during storage (Marschner 1995). This is particularly concerned in the case of fleshy fruits with their typically low Ca²⁺ levels. Application of Ca²⁺ to soils seems to be of questionable value (Lopez-Lefebvre et al. 2001); however, supplemental Ca²⁺ applied immediately before or just after harvest has been shown to maintain cell turgor, plasma membrane integrity, and fruit

firmness and extend storage life (Gerasopoulos et al. 1996).

The increase in the bioavailability of Ca²⁺ in vegetables could enhance their nutritional value and also improve yield (Marschner 1995). One molecular-genetic approach to alter Ca²⁺ levels in plants is to engineer a high expression of Ca²⁺ transporters and Ca²⁺-binding proteins (Hirschi 1999; Wyatt et al. 2002). Increased expression of a modified vacuolar Ca²⁺ antiporter, cation exchanger 1 (*CAX1*), in plants causes dramatic increases in Ca²⁺ when compared to wild-type plants (Hirschi 1999; Park et al. 2005b). In contrast, the transgenic crops ectopically expressing *sCAX1* displayed alterations in plant development and morphology, including increased incidence of blossom-end rot or tip-burn although the transgenic plants had dramatically elevated [Ca²⁺] in plants (Hirschi 1999; Park et al. 2004).

Arabidopsis (*Arabidopsis thaliana*) appears to have up to 12 putative *CAX* transporters (Mäser et al. 2001). Like *sCAX1*, *sCAX2* and *sCAX4* can function in yeast as H⁺/Ca²⁺ exchangers (Cheng et al. 2002). Park et al. (2005b) reported that the

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CAX4-expressing tomatoes show modest increases in Ca^{2+} levels and shelf life but no deleterious effects on plant growth and development. However, the specific transport properties of numerous CAX transporters, including *CAX4*, have not been examined widely.

In this study, we compare the effect of *CAX4* expression in tomato using a domestic cultivar as a method to determine if utilizing full-length CAX expression may be a means to reduce deleterious phenotypes. This study suggests that modulation of Ca^{2+} transporters could make an important contribution toward increasing the value of various agriculturally important crops.

Materials and methods

Plant material, transformation, and growth conditions

Seeds of tomato (*Lycopersicon esculentum* Mill.) inbred Tamnara, a domestic cultivar bred by National Institute of Horticultural and Herbal Science, were disinfected. Seeds were germinated on a Murashige and Skoog (1962) basic salt medium with 30 g/L sucrose, pH 5.7, and solidified using 8 g/L TC agar (Sigma-Aldrich, St. Louis, USA). Tomato transformation was performed via *Agrobacterium*-mediated transformation method using cotyledon and hypocotyl explants as described (Figure 1; Park et al. 2005b). Explants were isolated and cultured onto the preculture media (MS basic salt medium with 1.0 mg/L BAP, 0.1 mg/L NAA, 30 g/L sucrose, pH 5.7, solidified by 8 g/L TC agar) for one day. After preculture, the explants were immediately dipped into an *Agrobacterium* culture, slightly blotted and re-cultured on the same media for a 3-day co-cultivation period. The explants were then transferred to the fresh media of different plant growth regulator combination

(2.0 mg/L zeatin and 0.1 mg/L IAA) containing 100 mg/L kanamycin with 500 mg/L Clavamox™ (amoxicillin trihydrate, Smithkline Beecham, West Chester, PA) to control *Agrobacterium* growth. The cultures were maintained at 25°C under a 16-h photoperiod. After 6 to 8 weeks (subcultured once at 3–4 weeks), regenerated shoots were transferred to rooting medium for 6 weeks, and then established in soil.

Agrobacterium strain and plasmids

Agrobacterium tumefaciens LBA 4404 (Hoekema et al. 1983) carrying the binary vector pBI121 was used for the transformations. The plasmid was reconstructed with the T-DNA consisted of *nos-pro/nptIII/nos-ter/35S/CAX4/nos-ter* (Park et al. 2005b), and then introduced into *A. tumefaciens* using the freeze-thaw method (Holsters et al. 1978). The bacteria were grown for 2 days in YEP medium [10 g/L yeast extract (Becton, Dickinson and Company, Sparks, USA), 10 g/L peptone (Becton, Dickinson and Company, Sparks, USA), 5 g/L NaCl (Sigma-Aldrich, St. Louis, USA)] containing 50 mg/L kanamycin monosulfate (Sigma-Aldrich, St. Louis, USA) and 50 mg/L rifampicin (Sigma-Aldrich, St. Louis, USA) at 28°C on a rotary shaker (220 rpm) until an $\text{OD}_{600}=1.0$ was obtained.

DNA isolation, PCR and Southern blot analyses

Tomato genomic DNA was extracted from leaf tissue as previously described (Paterson et al. 1983). For PCR amplification of *nptIII* gene, one set of primers (5'-GAGGCTATTC GGCTATGACTG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3') was used. The conditions for amplification were 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 60°C for 45 s, 72°C for 1 min, and 72°C for 5 min. PCR products were run on 1.4% agarose gels and stained with ethidium bromide. For a Southern blot analysis on the randomly selected PCR-positive plants, DNA (5–10 mg) was digested with *XbaI* and separated by electrophoresis and blotted onto a nylon membrane (Zeta-probe GT membrane, Bio-Rad Laboratories) according to the manufacturer's instructions. The probe for the *CAX4* gene was isolated from a *SaI* (1.2 kb) restriction fragment of the pBlue-*CAX4* plasmid (Cheng et al. 2002). The membranes

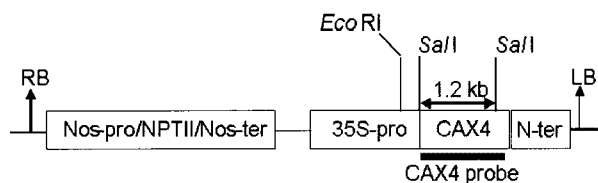


Figure 1. T-DNA regions of pCaMV35S::CAX4. RB, Right border; LB, left border; Nos-pro, nopaline synthase promoter; NPTIII, neomycin phosphotransferase; Nos-ter, nopaline synthase terminator; 35Spro, CaMV 35S promoter; CAX4, cation exchanger 4; N-ter, nopaline synthase terminator.

were pre-hybridized overnight at 65°C in 7% SDS and 0.25 M Na₂HPO₄, and then hybridized overnight at 65°C in the same solution containing the probe labeled with ³²P-dCTP using NEBlot kit (NEB BioLabs). Membranes were washed twice for 30 min each with 20 mM Na₂HPO₄ and 5% SDS at 65°C and then washed twice again for 30 min each with 20 mM Na₂HPO₄ and 1% SDS at 65°C. Membranes were exposed to X-ray film.

RNA isolation and Northern blot analysis

Total RNA was extracted from green fruit tissues and leaves using RNeasy plant kits (Qiagen) according to the manufacturer's instructions. Total RNA (7 mg) was separated on a 1.2% agarose gel containing 1.5% formaldehyde, blotted onto a Zeta-Probe GT membrane according to the manufacturer's instructions. Hybridization and washing were as previously described in Southern-blot analysis.

Ca²⁺ and mineral analysis

Fruit Ca²⁺ and mineral analysis was performed at 20 d after breaker stage, and the fruits (pooled at least five-fruit batches) were dried at 70°C for 4 d. A total of 0.25 g (dry weight) of

fruits was digested for analysis (Feagley et al. 1994). Total Ca²⁺ and mineral content per gram of dry weight was determined by inductively coupled plasma emission spectrophotometry (Spectro, <http://www.spectro.com>).

Blossom end rot (BER) occurrence analysis

After segregation analysis on T₂ seeds from self-pollinated T₁ plant lines (showing a segregation pattern of 3:1 on kanamycin medium), fruit from each of 10 homozygous T₂ lines was selected. All CAX4-expressing tomatoes were grown and ripened at 22°C to 24°C in the greenhouse. Observation on fruit number and percentage of incidences of BER were recorded on all fruits from all plant lines selected in this study.

Results and discussion

Agrobacterium-mediated transformation and nucleic acid analysis

After cocultivation of tomato cotyledon and hypocotyl explants with the bacteria for 3 days, some cotyledon and hypocotyl explants showed adventitious shoot formation with a range of 1.7-3.5 shoots per explant within 4-5 weeks (Figure

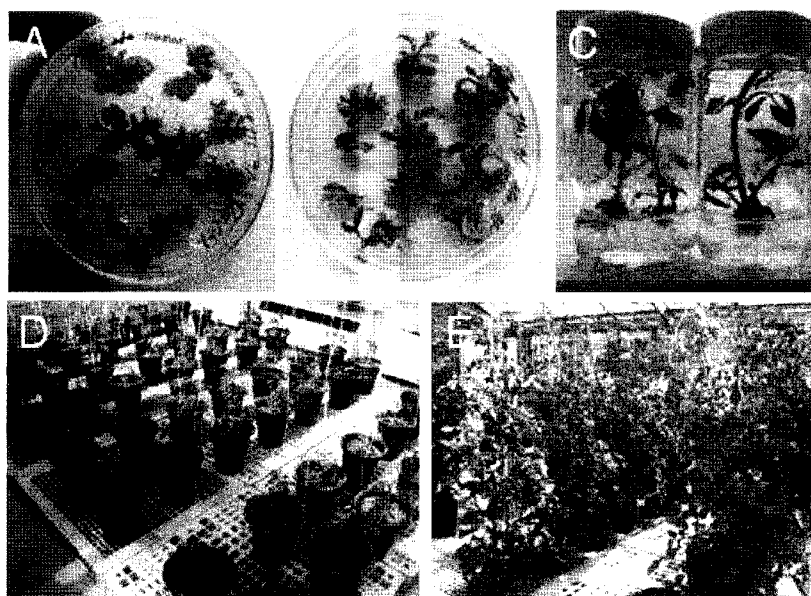


Figure 2. *Agrobacterium*-mediated transformation and phenotypes of CAX4-expressing tomato plants. Adventitious shoot formation from hypocotyls (A) and cotyledon explants (B) of tomato cultivar Tamnara. C, Rooted plantlets on a MS selection medium with 100 mg/L kanamycin. D, Growth of the CAX4-expressing plants after 3 weeks in the greenhouse. E, Fruit setting from the CAX4-expressing plant after 8 weeks in the greenhouse.

2A and 2B). These regenerated shoots were rooted and were also successfully acclimated in the greenhouse (Figure 2C and 2D). These putative transgenic plants did not perturb the morphology, growth, or fruit set (Figure 2E). We have generated twenty one putative transgenic events which derived from different experiment batches or explants. First, we examined the presence of the transgenes by PCR analysis. It demonstrated that fourteen T₀ plants among them possessed an integrated gene for kanamycin resistance (*nptII*) (Figure 3A, part of the data was shown). Seven randomly selected PCR-positive transformants were subjected to a Southern blot analysis for *CAX4* to confirm independent integration and determine copy number. As demonstrated in Figure 3B, the transgenic tomato lines contained various copy numbers of the *CAX4* construct. The lines we have termed TCT-2, TCT-5, TCT-11, and TCT-15 appeared to contain single insertions.

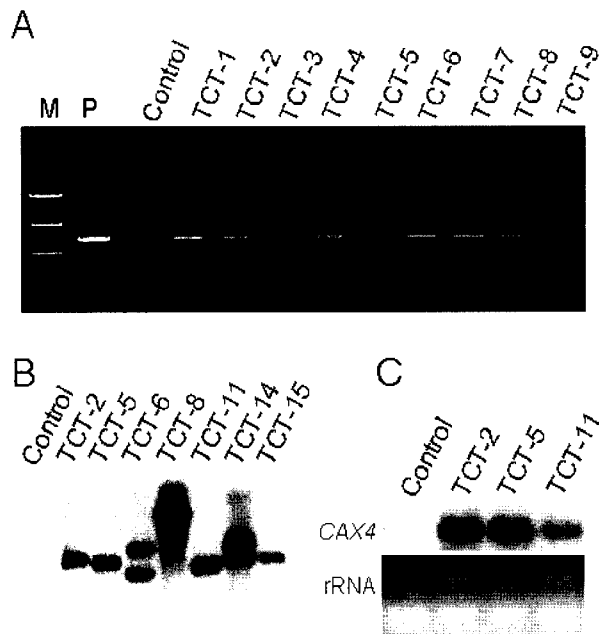


Figure 3. Molecular analyses of *CAX4*-expressing tomatoes. A, PCR detection of *nptII* genes in the putative transgenic tomato plants. Expected 650 bp fragments of *nptII* were amplified in most of tested plantlets after acclimatization. Lanes M, molecular size marker; P, positive control (plasmid DNA); Control, wild-type tomato; TCT1 through TCT9, putative transgenic tomato plants. B, Southern-blot analysis of transgenic tomato plants. Five to 10 mg of tomato genomic DNA were digested with *Xba*I, and hybridized with the *CAX4* cDNA probe. C, Northern-blot analysis of transgenic tomato plants. Ten micrograms of total RNA from expanded leaves and green fruits were hybridized with *CAX4* cDNA probe. Ethidium bromide-stained rRNA (bottom) is shown as a loading control.

RNA gel blot documented that the *CAX4* transcripts accumulated in all of the transgenic lines (Figure 3C, part of the data was shown). The inability to detect *CAX4* homology in the wild-type lines by Southern or Northern analysis may be due to the stringency of hybridization used in this study. Three T₀ transgenic lines (TCT-2, TCT-5, and TCT-11) showing one copy number of the *CAX4* gene were selected and subjected to further analysis of Ca²⁺ accumulation and shelf life in *CAX4*-expressing fruits.

Phenotypes of *CAX4*-expressing tomatoes

While the *sCAX1*-expressing transgenic tomatoes were sensitive to Ca²⁺ deficiency and showed Ca²⁺ deficiency-like symptoms that were suppressed by addition of Ca²⁺ (Hirschi 1999; Park et al. 2004), the *CAX4*-expressing lines were not sensitive to Ca²⁺ deficiency and did not require any additional Ca²⁺ supplementation for normal growth. Although the deregulated *sCAX1*-expressing plants showed Ca²⁺ deficiency-like symptoms (i.e. necrotic lesions) that were suppressed by the addition of Ca²⁺ (Hirschi 1999; Park et al. 2004, 2005a, b), the *CAX4*-expressing tomatoes did not alter their morphology or growth characteristics (Park et al. 2005b). In addition, the *CAX4*-expressing tomatoes did not require any additional Ca²⁺ supplementation for normal growth like as shown the previous studies (Park et al. 2005b; Kim et al. 2006). These results infer that the ectopic expression of CAX exchangers accompany a distinguished phenotypes depending upon the existence of auto-inhibitory domain of CAX.

Ionic alteration in *CAX4*-expressing tomato plants

The fruits of *CAX4*-expressing T₀ tomatoes showed a 63% to 71% more increase in Ca²⁺ level than wild-types (Figure 4), while there were no differences in other metal ions (Cu²⁺, Fe²⁺, Mg²⁺, and Mn²⁺) level in all lines analyzed (Table 1). In carrots and potatoes, the expression of *sCAX1* has been shown to increase the total Ca²⁺ accumulation in aerial portion of the plants, but this causes modest changes in altered morphology as well (Park et al. 2004; 2005b). The expression of *sCAX2* in tobacco and *sCAX2B* in potato, however, did not appear to

alter plant growth. This may be in part to low Ca^{2+} sequestration into the vacuole (Hirschi et al. 2000; Kim et al. 2006). In previous study, the *CAX4*-expressing tomato plants showed no alteration of morphological phenotypes meanwhile some *sCAX1*-expressing tomato plants showed Ca^{2+} deficiency-like symptoms (i.e. necrotic lesions) (Park et al. 2005b). This may

be not only in part to moderate calcium accumulation in the *CAX4*-expressing tomato plants but may correlate with the absence in accumulation of various transition metals (Marschner, 1995). It is also worth noting that increased $\text{H}^+/\text{Ca}^{2+}$ transport activity may activate other proton-mediated transporters (Cheng et al. 2005) to cause alterations in plant growth.

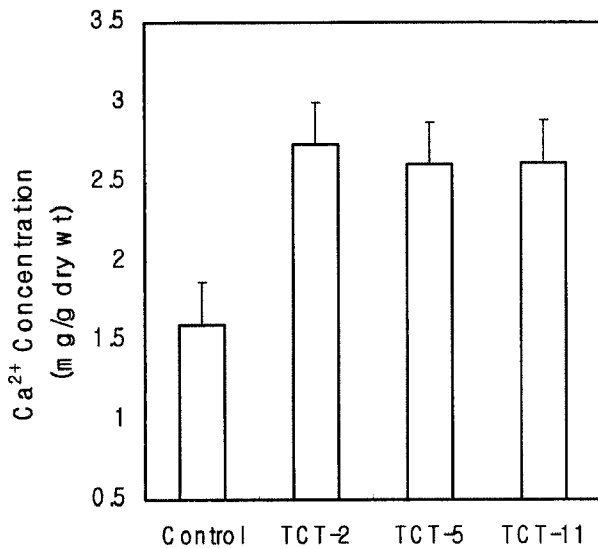


Figure 4. Concentrations of Ca^{2+} in fruits of *CAX4*-expressing plants and wild-type. Fruit Ca^{2+} analysis was performed at 20 days after breaker stage. Total Ca^{2+} contents of fruits (pooled at least five-fruit batches) were determined by inductively coupled plasma emission spectrophotometer.

BER occurrence in *CAX4*-expressing tomato plants

No significant differences were observed in BER incidence between the fruits of *CAX4*-expressing tomato plants (BER ratio; $28 \pm 5\%$) and the ones of wild-type plants (BER ratio; $24 \pm 6\%$) (Table 2). It is widely accepted that BER is caused by calcium deficiency at the distal end of tomato fruits during the initial stage of fruit development within a few weeks after anthesis (Bangerth 1979). The appearance of physiological disorders caused by calcium deficiency may be closely correlated to transport systems of Ca^{2+} . There are likely to be two pathways of calcium movement through the symplast and through the apoplast in plant tissues (White 2001). Cellular calcium homeostasis is maintained by an ensemble of calcium transport proteins such as Ca^{2+} -ATPases, $\text{Ca}^{2+}/\text{H}^+$ antiporters, and Ca^{2+} channels (Bush 1995). It has been suggested that decreases in cytosolic and apoplastic Ca^{2+} levels may disrupt Ca^{2+} -signaling

Table 1 Concentrations of cations in fruits of wild-type and *CAX4*-expressing tomato plants

Lines	Concentration of cation			
	Mg^{2+} (mg/g dry wt.)	Fe^{2+} ($\mu\text{g/g}$ dry wt.)	Mn^{2+} ($\mu\text{g/g}$ dry wt.)	Cu^{2+} ($\mu\text{g/g}$ dry wt.)
Wild-type	2.3 a ²	36.1a	33.4a	11.2a
TCT-2	2.4 a	39.1a	36.5a	13.5a
TCT-5	2.1 a	35.3a	34.3a	12.4a
TCT-11	2.2 a	38.2a	34.8a	13.2a

²Means with different letters indicate significant differences at $P \leq 0.05$ by Duncan's Multiple Range Test. Fruit cations analysis was performed at 20 d after breaker stage. Total cations contents of fruits (pooled at least five-fruit batches) were determined by inductively coupled plasma emission spectrophotometer.

Table 2 Blossom end rot (BER) occurrence in fruits of wild-type and *CAX4*-expressing tomato plants

Lines	% of blossom end rot (BER) fruit
Wild-type	$24 \pm 6a^2$
T ₂ progenies of <i>CAX4</i> -expressing tomato	$28 \pm 5a$

²Means with different letters indicate significant differences at $P \leq 0.05$ by Duncan's Multiple Range Test. The incidence of BER in fruit from each of 10 homozygous T₂ lines was recorded at 20 d after breaker stage.

processes, membrane integrity, and normal cell wall development, leading to cell death and thus the occurrence of BER (Ho and White 2005; Park et al. 2005b). In this study, despite of the increased Ca^{2+} in tomato fruits by the sequestration of Ca^{2+} from cytosol into vacuole which feasibly disturb the cytosolic Ca^{2+} homeostasis, the equivalent of BER incidence in *CAX4*-expressing tomatoes deserves attention. We expect that the increased Ca^{2+} in the tomato fruit is due to a significant increase in vacuolar Ca^{2+} level within the rapidly expanding fruit by the enhanced and/or unregulated vacuolar $\text{Ca}^{2+}/\text{H}^+$ antiporter (*CAX4*) activity. Meanwhile, one possible explanation for the equivalent of BER in *CAX4*-expressing tomato lines might be to alter the activities of other cellular Ca^{2+} -related machineries for keeping up the cytosolic Ca^{2+} homeostasis.

Here we have used full-length *CAX4* containing the entire putative N-terminal autoinhibitory domain. *CAX4* is 54% identical to *CAX1* at the amino acid level, and previous work suggests that repositioning of the N-terminus in this transporter confers Ca^{2+} transport in yeast assays (Cheng et al. 2002). In this report, we have demonstrated the ability to increase Ca^{2+} levels in tomatoes through heightened activity of a Ca^{2+} transporter (*CAX4*). We have also demonstrated here that expression of the $\text{H}^+/\text{Ca}^{2+}$ transporter can increase fruit Ca^{2+} levels and with no accumulation of other metallic cations as using a domestic tomato cultivar Tamnara.

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