Calcium stabilizes cell membranes by connecting adjacent polar head groups of membrane lipids (Legge et al., 1982; Palta, 1996; Hirschi, 2004). It also protects membranes from adverse effects of stress such as salinity (Cramer et al., 1985), freezing injury (Arora and Palta, 1988), and heat stress (Tawfik et al., 1996; Kleinhenz and Palta, 2002; Saidi et al., 2009). Calcium is also a component of cell walls that forms stiff gels through Ca$^{2+}$–mediated crosslinking of its carboxyl groups through ionic and coordinate bonds with a pectin component called homogalacturonan or polygalacturonic acid (Cosgrove, 2005; Palta, 1996).

Calcium homeostasis maintains the concentration of extracellular Ca$^{2+}$ in the millimolar range, whereas the cytoplasmic concentration of Ca$^{2+}$ is in the nanomolar to micromolar range (Kauss, 1987; Gilroy et al., 1993). Calcium antiporters and efflux pumps are important to maintain the cytoplasmic calcium at low levels and restoring the normal Ca$^{2+}$ levels after perturbation (Tuteja and Mahajan, 2007). A constant supply of Ca$^{2+}$ in the range of 1 to 10 mM is required to maintain normal growth and development at the whole-plant level (Epstein, 1972; Clarkson and Hanson, 1980). Calcium moves with water mainly by the apoplast and its redistribution within the plant is very low (Bangerth, 1979; Clarkson 1984; Busse and Palta, 2006). In plant tissues that are deficient in Ca$^{2+}$, cell walls disintegrate and tissue

**CAX1 Vacuolar Antiporter Overexpression in Potato Results in Calcium Deficiency in Leaves and Tubers by Sequestering Calcium as Calcium Oxalate**

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**ABSTRACT**

Recent studies show that overexpression of a short version of the cation exchanger 1 gene (sCAX1) can cause Ca$^{2+}$ deficiency symptoms in tomato (Solanum lycopersicum L.). However, the Ca$^{2+}$ deficiency in relation to the overexpression of this gene has not been investigated in potato (Solanum tuberosum L.). The objective of our study was to investigate the production of known Ca$^{2+}$ deficiency symptoms in potato in relation to the overexpression of sCAX1. Plantlets of S. tuberosum cultivar ‘Atlantic’ overexpressing the sCAX1 gene were produced using Agrobacterium tumefaciens. Transgenic plants grown with normal amounts of Ca$^{2+}$ under in vitro or greenhouse conditions showed known Ca$^{2+}$ deficiency symptoms in potato plants such as shoot tips damage and leaf margin necrosis, as well as tuber internal defects (hollow heart). Growing the transgenic plants with higher amounts of Ca$^{2+}$ in the media or soil nutrient solution mitigated these symptoms. These results support the notion that both shoot tip necrosis and hollow heart are associated with Ca$^{2+}$ deficiency. There was abundance of calcium oxalate (CaC$_2$O$_4$) crystals present only in the transgenic plants suggesting that these plants sequester Ca$^{2+}$ in the form of CaC$_2$O$_4$ in the vacuoles of transgenic plants, reducing Ca$^{2+}$ in the other pools. Since both shoot tip necrosis and hollow heart are known to be associated with poor cell wall health, our results suggest that CAX1 is a regulator of Ca$^{2+}$ in the cell wall. In support of this concept, we found reduced cell wall biomass in the transgenic plants compared with the wild type.
collapses, resulting in necrosis (Kirkby and Pilbeam, 1984; Marschner, 1995; Busse et al., 2008).

The H\(^+\)/Ca\(^{2+}\) antiporter 1, CAX1, is a tonoplast Ca\(^{2+}\) antiporter identified and cloned from *Arabidopsis thaliana* (L.) Heynh., by suppressing yeast mutants’ defective in vacuolar Ca\(^{2+}\) transport (Hirschi et al., 1996). Several CAX antiporters have been identified in *A. thaliana* with different ion specificities such as CAX2 that transports heavy metals (Hirschi et al., 2000), CAX3 that transports Ca\(^{2+}\) mainly in roots (Manohar et al., 2011), and CAX4 (Cheng et al., 2002), among others. The N-terminal regulatory region of CAX1 acts as an autoinhibitory domain for H\(^+\)/Ca\(^{2+}\) transport activity when expressed in yeast (Pittman and Hirschi, 2001; Pittman et al., 2002). This region was removed to generate a deregulated short version, denominated sCAX1 (Cheng et al., 2005). The overexpression of sCAX1 in tobacco (*Nicotiana tabacum* L.; Hirschi, 1999), carrots (*Daucus carota* L.; Park et al., 2004), potato (Park et al., 2005a), and tomato (Park et al., 2005b) has shown to increase Ca\(^{2+}\) content in leaves, roots, tubers, and fruits, respectively.

The *A. thaliana* mutants lacking CAX1 (cax1) showed a reduction of Ca\(^{2+}\)/H\(^+\) antiporter activity, a reduction in tonoplast V-type H\(^{+}\)-translocating ATPase activity, an increase in tonoplast Ca\(^{2+}\)-ATPase activity, and the lack of CAX1 was compensated by increased expression of CAX3 and CAX4 (Cheng et al., 2003). Also, cax1 mutants have shown characteristics of plants that grow in serpentine soils such as greater tolerance for low Ca\(^{2+}\), increased tolerance to Mg\(^{2+}\), higher Mg\(^{2+}\) requirement for maximum growth, and Ca\(^{2+}\) exclusion from leaves (Bradshaw, 2005). In addition, cax1/cax3 double mutants displayed a severe reduction in growth, including leaf tip and flower necrosis and pronounced sensitivity to exogenous Ca\(^{2+}\) and other ions (Cheng et al., 2005). CAX1 has been recognized as a key regulator of the apoplastic Ca\(^{2+}\) concentration. CAX1 keeps apoplastic Ca\(^{2+}\) low through compartmentation into mesophyll vacuoles that is essential for normal plant function and productivity (Conn et al., 2011). The unbound extracellular Ca\(^{2+}\) must be maintained in equilibrium across the apoplast–symplast boundary. In leaves, the vacuole serves as the reservoir for excess accumulation of Ca\(^{2+}\) rather than the cell wall (Robertson, 2013). Park et al. (2005a) transformed the potato cultivar ‘Russet Norkotah’ with a small version of CAX1 and reported that increased levels of CAX1 do not appear to alter potato growth and development, nor tuber morphology or yield. They also reported an increased Ca\(^{2+}\) concentration in various sections of the tuber in the transgenic. On the other hand, some studies have shown that an overexpression of sCAX1 in tomato can result in Ca\(^{2+}\) deficiency symptoms such as blossom–end rot (de Freitas et al., 2011). These results are consistent with the observation that overexpression of CAX1 can lead to lower apoplastic Ca\(^{2+}\) that is essential for normal plant function (Conn et al., 2011).

In potato, several studies have established Ca\(^{2+}\)-related deficiency symptoms in tubers and leaf tissue (Palta, 1996; Busse et al., 2008). For example, a Ca\(^{2+}\) deficiency in the tuber tissue leads to the development of a cavity in the center of the growing tuber known as hollow heart (Rex and Mazza, 1989; Palta, 1996). Several physiological disorders, including hollow heart, in tuber tissue caused by Ca\(^{2+}\) deficiency can be mitigated by increasing tuber Ca\(^{2+}\) concentration through fertilization (Kleinhenz et al., 1999; Karlsson et al., 2006; Ozgen et al., 2006). Results of recent studies outlined above would suggest that overexpression of CAX1 in potato could result in some of these Ca\(^{2+}\) deficiency–related physiological disorders in tuber tissue. In the present study, we tested this idea.

It is also known that Ca\(^{2+}\) deficiency in many plants, including potatoes, can cause shoot tip injury that leads to tip necrosis and death of the main shoot (Sha et al., 1985, Busse et al., 2008; Ozgen et al., 2011). This shoot tip injury has been shown to result from collapse of expanding subapical cell walls due to lack of Ca\(^{2+}\) (Busse et al., 2008). Again, recent studies outlined above would suggest that overexpression of CAX1 in potato could result in shoot tip necrosis. In the present study, we also tested this idea.

We first reported potato plants overexpressing sCAX1 suffering from Ca\(^{2+}\) deficiency (Zorrilla and Palta, 2012). Here, we present detailed information describing the phenotypic characteristics associated with the overexpression of sCAX1 in potatoes, evaluate the effects on plant health, tuber internal defects, shoot tip necrosis, and Ca\(^{2+}\) content, and identify the presence of calcium oxalate (CaC\(_2\)O\(_4\)) crystals.

**MATERIALS AND METHODS**

**Plant Material and Transformation Constructs**

In vitro-grown plantlets, 5 to 6 wk old, of the potato cultivar ‘Atlantic’ were transformed with the *Agrobacterium tumefaciens* LBA4404 harboring the small version of the CAX1 gene from *Arabidopsis thaliana* (Park et al., 2005a). Two gene constructs developed by Park et al. (2005a) were used for transformation, one carrying the CaMV35S promoter CaMV35S::sCAXI, and other carrying the cdc2a promoter, cdc2a::sCAXI. The CaMV 35S and the cdc2a promoters were chosen due to their high and non-tissue-specific transcription (Odell et al., 1985; Doerner et al., 1996). The Atlantic transgenic lines were named using AT1 for lines carrying the CaMV35S::sCAXI construct and AT2 for lines carrying the cdc2a::sCAXI construct. The numbers following indicate plate number, explant number, and shoot number separated by an underscore. The Russet Norkotah transgenic lines CAX1 #34, CAX1 #36 K-3-1, CAX1 #36 K-3-2, and CAX1 #49 and a wild-type line of Russet Norkotah RN#12 generated by Park et al. (2005a) were kindly provided by Kendall Hirschi from Texas A&M University, College Station, TX. All wild-type and transgenic lines were maintained in tissue culture using Murashige–Skoog (MS) basal media (Murashige and Skoog, 1962) modified with CaCl\(_2\) as the source of Ca\(^{2+}\). Varying amounts of Ca\(^{2+}\) were used for maintenance depending on the optimal for each line.
Potato Transformation

Stem cuttings of 1 to 2 cm long were co-cultured with the Agrobacterium strains carrying either the CaMV35S::sCAX1 or the cdc2a::sCAX1 constructs in a culture media (ZIG) containing 5 mg L\(^{-1}\) trans-zeaatin riboside, 0.01 mg L\(^{-1}\) indol acetic acid, and 0.2 mg L\(^{-1}\) gibberellic acid. After 4 to 6 d of co-cultivation under low light, stem cuttings were transferred to another media (ZIG\++) containing ZIG media with 50 mg L\(^{-1}\) kanamycin (selective agent) and 100 mg L\(^{-1}\) cefotaxime (Agrobacterium killing agent). Then, they were transferred to ZIG ++ media every 2 wk until some shoots appeared in the callus formed at the edge of the stem cuttings. Shoots of at least 2 cm long were transferred to MS basal media with 50 mg L\(^{-1}\) kanamycin (MS+) or to media (MS++) containing MS with 100 mg L\(^{-1}\) cefotaxime and 50 mg L\(^{-1}\) kanamycin if any signs of Agrobacterium growth were observed. Shoots that developed a strong root system were maintained as putative transgenic.

PCR

Genomic DNA was isolated from plantlets using an extraction protocol based on a cetyltrimethylammonium bromide (CTAB) 2× buffer (Doyle and Doyle, 1990), with a tissue lysis step of 20 s using 64-mm ceramic beads performed with a Bead Beater (Biospec) or a Fast Prep-12 instrument (MP Biomedicals), and treated with RNase A (Invitrogen) after isolation. The presence of the transgene was determined by polymerase chain reaction (PCR) of the neomycin phosphotransferase (NPT II) marker gene. The sequences of the primers used for the NPT II gene were 5′-AGC CAA CGC TAT GTC CTG AT-3′ and 5′-GAA GGG ACT GGC TGC TAT TG-3′ (Genbank accession U09365). The presence of a 370-bp band indicated that the plant had been transformed.

PCR Efficiency and Copy Number Determination

Transgene copy number determination based on real-time PCR is a fast, inexpensive, and high-throughput alternative (Yuan et al., 2007). Primers that target the CAXI gene, the NPT II gene, and single-copy genes including the granule-bound starch (GBSS, also called waxy) gene and EF1-α were used to determine their PCR efficiency. The primer sequences tested to determine their PCR efficiency. The primer sequences used were 5′-GAAGAAATCGCTCCACTTGC-3′ and 5′-GAAGAAAACCAAT-3′ to amplify a 387-bp fragment of the GBSS gene (Genebank accession X83220), the 5′-ATTGGAAACGGATATGCTCCA-3′ and 5′-TCTCCACAGCTTGCAGCTTGC-3′ to amplify a 370-bp fragment of the EF1-α gene (Nicot et al., 2005), the 5′-AGACAATCGGCTCCTGAT-3′ and 5′-AGTGACACGATCCGAGCAGC-3′ to amplify a 370-bp fragment of the NPT II gene (GenBank accession U09365), and the 5′-GCAACAGCAAGCAGCTTGCAGC-3′ and 5′-AATCCACGGATATGCTCCA-3′ to amplify a 370-bp fragment of the CAXI gene (TAIR: AT2G38170). The efficiency of the PCR reactions in this study was estimated using the R\(^2\) and the slope of the linear relationship between the threshold cycle (Ct) values vs. the log\(_2\)–transformed DNA amounts. The Ct values are obtained from a CFX96 real-time detection system (BIO–RAD). A slope close to −1 and R\(^2\) close to 100% is considered a good indicator of PCR efficiency (Saunders, 2004). The DNA concentration was determined using a nanodrop ND-2000 (Thermo Scientific). Template amounts of 50, 100, 200, and 400 ng of the same transgenic clone in triplicate were used for the calibration curve. The PCR reaction mix contained 1× Ex-Taq buffer (Takara), 3 mM MgCl\(_2\), 300 μM deoxynucleotides, 0.8 μM of each primer, 0.8x Evagreen (Biotium), and 0.125 units of Takara Ex-Taq Hot Start version in a final volume of 25 μL.

For each transgenic line, two replicates of the real-time reactions for both genes were performed in different wells of the same plate. One line was used in all plates as a plate variation control because not all lines could fit in a single plate. A mixed model that accounts for all the sources of variation was used to determine the predicted Ct values for each line and gene.

Transcripts Quantification

The tips of two in vitro-cultured plantlets measuring ~1.5 cm were removed, quick frozen in liquid N, and stored at −70°C until RNA extraction. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen), and the contaminating DNA was removed using the Turbo DNA-free kit (Ambion) according to the manufacturer’s recommendations. The complementary DNA (cDNA) was produced using the Superscript III First Strand cDNA Synthesis System (Promega) from 1 μg of purified total RNA according to the manufacturer’s specifications. The RNA and cDNA concentrations were determined using a nanodrop ND-2000 (Thermo Scientific). The PCR efficiency of the quantitative PCR reaction was tested for the housekeeping gene EF1-α and the CAXI gene. EF1-α was chosen as the control housekeeping gene because it has been reported as the most stable under biotic and abiotic stress in potato (Nicot et al., 2005). The PCR reaction and primers were same as the copy number assay. The PCR efficiency was high for the EF1-α (slope < −0.98, R\(^2\) > 0.91) and sCAXI (slope < −0.99, R\(^2\) > 0.96) genes used for the quantitative PCR. A mixed model that accounts for main sources of variation was used to determine gene expression.

In Vitro Evaluations

Using node cuttings, each transgenic clone was grown at standard Ca\(^{2+}\) concentration present in MS media (Murashige and Skoog, 1962), also called the normal Ca\(^{2+}\) treatment: 3 mM Ca\(^{2+}\) in the form of CaCl\(_2\), as well as high Ca\(^{2+}\) treatments: 10, 15, and 30 mM Ca\(^{2+}\) in the form of CaCl\(_2\). The transgenic clones were evaluated using a randomized block design with three replications of each transgenic line and the wild type. Each replication contained six to eight plants. Plants were grown under in vitro conditions for 5 to 6 wk. A set of three transgenic lines with a single copy of sCAXI was evaluated in these experiments: AT1_02_01_01, AT1_08_02_01, and AT2_01_09_01; these lines were renamed as Line 1, Line 2, and Line 3, respectively.

The appearance of necrosis, malformation, or multiple shoots was recorded as shoot tip damage. The presence or absence of shoot tip damage was registered for each plant. The incidence of shoot tip damage was estimated as the average presence of tip damage. The probability of having shoot tip damage was estimated for each line. In vitro plantlet height and weight was measured in centimeters and grams, respectively, using only the aboveground portion of plantlets.
Greenhouse Evaluations

Plants were grown in the Walnut Street greenhouses located at the University of Wisconsin–Madison campus. The growing mix contained vermiculite, perlite, and peat moss (1:1:1, volume ratio). The mix was placed in 4-L pots up to three-fourths of the pot volume and washed several times with tap water to remove excess water-soluble nutrients including Ca\(^{2+}\) present in the growing mix. The pH of the growing mix was 5 after washing. Plants were fertilized once per day with 500 mL of a 0.52-g L\(^{-1}\) Peat-Lite Special 20–10–20 N–P–K solution containing a full range of essential micronutrients (Peter’s Professional) made with tap water that contained 1.75 mM Ca\(^{2+}\) (70 mg L\(^{-1}\)). Environmental conditions in the greenhouse were 20°C during the day and 15°C during the night, with 16-h light/8-hour dark photoperiod, and light intensity at the top of the plants was, on average, 600 μmol m\(^{-2}\) s\(^{-1}\). Light was supplemented with high-intensity discharge lamps when needed. The experimental design was a randomized block design with three replications per line per treatment.

The greenhouse experiments were performed using a set of three transgenic Atlantic lines with a single copy of sCAXI—Line 1, Line 2, and Line 3—and Atlantic. The treatments applied were 1, 10, and 15 mM supplied in the form of CaCl\(_2\). For normal growth, a constant supply of 1 to 10 mM Ca\(^{2+}\) is considered adequate (Epstein, 1972; Clarkson and Hanson, 1980) to maintain normal growth and development. We included a 15 mM treatment to see if Ca\(^{2+}\) deficiency in the transgenic plants were mitigated at higher Ca\(^{2+}\) concentrations. Tubers were harvested after 3 mo. Tuber total Ca\(^{2+}\) on a dry weight basis and tuber internal defects were evaluated using 5 to 10 tubers from each plant (replicate), with two to four replicates per treatment. The probability of having internal defects was estimated for each line using a generalized linear model based on the average number of tubers per plant that show hollow heart, a serious type of internal defect in which a cavity is formed in the center of the tuber.

Total Calcium (Plant and Tuber Tissues) Quantification

Tuber and in vitro shoot culture tissues were processed for Ca\(^{2+}\) concentration quantification. A protocol based on the method presented by Kratcke and Palta (1986) was used for extraction and quantification of Ca\(^{2+}\). Tuber tissue internal to the vascular ring were sampled from 20 1-mm-thick slices cut from the center of 5 to 16 tubers, depending on the size of the tubers. Tissue samples were either freeze dried or dried in an oven at 60°C. The amount of dried sample required depended on the type of tissue; 0.1 g was used for in vitro plants, and 0.5 g was used for tuber tissue. Samples were ground to pass a 20-mesh screen, ashed at 550°C, and Ca\(^{2+}\) was extracted using 5 mL 2 M HCl, filtered using an acid-treated filter paper. Then, 10 mL LaCl\(_3\) (2000 mg L\(^{-1}\)) was added to the flask and the volume was raised to 50 mL. Calcium was quantified using an atomic absorption spectrophotometer (Varian SpectrAA 55B, Agilent Technologies) and expressed as micrograms per gram of dry weight (μg g\(^{-1}\)).

Tuber Cell Wall Extraction

Cell walls were extracted from greenhouse-grown potato tubers using a protocol adapted from Hoff and Castro (1969). Tuber tissue internal to the vascular ring were sampled from 20 1-mm-thick slices cut from the center of 5 to 16 tubers, depending on the size of the tubers. Slices were rinsed in prechilled distilled–deionized water to remove the excess of starch and mopped dry in a mesh cloth before determining the fresh weight. The slices then were separated in two samples. One sample was used for total Ca\(^{2+}\) extraction and processed as described above. The other sample was frozen at −20°C immediately after sampling and used for cell wall extraction at later time. For cell wall extraction, the tissue was blended using a food blender in 100 mL of cold distilled–deionized water, sieved using a propylene mesh with 10-μm × 100-μm pores, and washed with 900 mL of cold distilled–deionized water to remove starch. The tuber tissue was disrupted using a sonicator (wave amplitude = 20) on an ice bath for a total of 40 cycles of 15 s sonication with 30 s cooling. The sonicated tissue was sieved using a propylene mesh with 100-μm × 100-μm pores and rinsed several times with cold distilled–deionized water, with a final rinse with 30 mL absolute ethanol. The tuber cell walls were frozen at −20°C until Ca\(^{2+}\) extraction. Tuber cell wall Ca\(^{2+}\) was extracted and processed as described above.

Calcium Oxalate Crystal Observation Using Polarized Light Microscopy

Calcium oxalate crystals were observed in leaf tissue using a light microscope (Olympus BX60) equipped with filters for polarized light capability in the Newcombe Imaging Center of the University of Wisconsin–Madison. Transverse sections of 100 to 200 μm were obtained from a leaflet of the third fully extended leaf using a vibratome sectioning system (Oxford). In this procedure, the sample is submerged in tap water and tissue sections are cut by a vibrating blade. The transversal sections were immersed in preboiled water at room temperature and subjected to vacuum for 5 min to remove air from the sample. Tap water was used throughout the sectioning to avoid cell injury. In addition, observations were also made on peels from the abaxial surface of leaf mid-ribs using forceps.

Leaf Margin Surface Morphology Evaluation Using Environmental Scanning Electron Microscopy

Morphology of leaf margin surfaces of transgenic and wild-type lines was observed using an environmental scanning electron microscope (ESEM FEI Quanta 200). Squares of 5 × 5 mm were sampled from leaf margins and immediately observed under the microscope. The sample parameters were set as follows: sample temperature = 5°C, accelerated voltage = 20 kV, spot size = 4, water vapor pressure = 677.3 Pa, and amplification = 500×.

Statistical Analysis

Linear models were used to determine copy number and gene expression. The linear model for copy number determination was: \(\text{Ct} = \log_2(\text{dna}) + \text{gene} + \text{line} + \text{plate} + \text{line} \times \text{gene}\), where \(\log_2(\text{dna})\) and gene are fixed-effect variables and line, plate, and line × gene were used as random-effect variables. The predicted Ct values were used to estimate copy number using a \(\Delta\Delta\text{Ct}\) method as described by Livak et al. (1995). An external
calibration curve was used to normalize for the variation in DNA concentration. The formula to estimate copy number is copy = \(2^{\Delta \Delta C_T}\), where \(\Delta C_T = (C_T_{EF1a} - C_T_{CAX1})_{unknown} - (C_T_{EF1a} - C_T_{CAX1})_{reference}\). The lines with known copy number were CAX1#34 (1 copy), CAX1#36 k-3-1 (1 copy), CAX1#36 k-3-2 (1 copy), and CAX1#49 (2 copies). The linear model for gene expression was: \(C_T = \text{line} + \text{gene} + \text{treatment} + \log_2(\text{cDNA}) + \text{line} \times \text{gene}\), where all variables were treated as fixed effects.

The relative difference between the quantity of transcripts of the reference gene EF1-a and the transgene sCAX1 was determined by the \(2^{\Delta \Delta C_T}\) method (Yuan et al., 2007). In addition, the relative difference of the quantity of transcripts among lines was estimated using the \(2^{\Delta \Delta C_T}\) method (Yuan et al., 2007). An internal standard curve generated with 25, 50, 100, and 200 ng of cDNA from the same clone was included in this evaluation.

The ANOVA was used to determine the significance of the effects of treatments and lines in the in vitro and greenhouse experiments for height, weight, and total Ca\(^{2+}\). The analysis of deviance, or ANODEV, was used to determine the significance of the effects of treatments and lines in the in vitro and greenhouse experiments for binomial data such as incidence of tip damage and internal defects.

The comparison between transgenic sCAX1 vs. wild-type lines was performed using an analysis of contrasts. For normal data (height, weight, and total Ca\(^{2+}\)), the means differences are nonsignificant if the confidence interval includes a value of zero.

The probability of having shoot tip damage or defective tubers at different treatments was estimated for each line using a generalized linear model for binomial data.

The statistical analyses and plots were obtained using the stats, lme4, graphics, and ggplot2 packages of R version 3.1.1 (R Development Core Team, 2013).

RESULTS

Transgenic Lines and Copy Number

Putatively transformed Atlantic lines were selected with the MS++ media that contains kanamycin and cefotaxime. DNA was extracted from these lines to test for the presence of the NPT II gene (Fig. 1). Forty transgenic lines, including 39 lines transformed with the CaMV35S::sCAX1 construct and one line with the ddc2a::sCAX1 construct, were obtained. From them, we were able to determine copy number of the transgene in 30 lines, including 29 lines transformed with the CaMV35S::sCAX1 construct and one line with the ddc2a::sCAX1 construct. In addition, four Russet Norkotah sCAX1 lines and their wild type were used for the evaluations as controls (Table 1).

Fourteen lines had one copy and 16 lines had more than one copy of the transgene. An initial in vitro experiment of the thirty Atlantic sCAX1 lines allowed the evaluation of phenotypic variability (Table 1). A set of three Atlantic sCAX1 lines that carry a single copy of sCAX1 (AT1_02_01_01 renamed Line 1, AT1_08_02_01 renamed Line 2, and AT2_01_09_01 renamed Line 3), two transgenic Russet Norkotah sCAX1 lines (CAX1#36 K-3-1 and CAX1#36 K-3-2), as well as Atlantic and Russet Norkotah wild-type lines, were selected to be used in further experiments. The comparison of the relative CAX1 gene expression in apical shoot tips of sCAX1 lines revealed that Line 3, the only Atlantic sCAX1 line carrying the ddc2a promoter, had almost double the expression compared with the other lines (Table 2).

Effects of Overexpressing sCAX1 on Shoot Tip Injury

Shoot tip damage is a necrosis and malformation of the shoot tips (Fig. 2A and 2B). This symptom was measured at normal and high Ca\(^{2+}\) treatments under in vitro and greenhouse conditions. The comparison between sCAX1 transgenic lines and their wild types at normal, 3 mM Ca\(^{2+}\) under in vitro conditions indicated that shoot tip damage is observed in the transgenic lines and absent in wild-type lines (Fig. 2A). A similar response was observed under greenhouse conditions; shoot tip damage was observed in the sCAX1 lines (Fig. 2C) but absent in the wild type (Fig. 2D) at the normal, 1 mM Ca\(^{2+}\) concentration in the nutrient solution.

Effects of Overexpressing sCAX1 on Leaf Morphology and Oxalate Presence under Greenhouse Conditions

The effect of overexpressing sCAX1 on the morphology of leaves was evaluated in plants grown under greenhouse conditions. When Ca\(^{2+}\) concentration in the nutrient...
Table 1. Variation among transgenic lines for incidence of shoot tip damage and tissue Ca\(^{2+}\) concentration in response to media Ca\(^{2+}\) concentration. Copy number of the sCAX1 transgene and concentration of Ca\(^{2+}\) in the media needed to maintain healthy plantlets in vitro are also indicated.

<table>
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<th>Lines</th>
<th>Promoter</th>
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<th>10 mM</th>
<th>30 mM</th>
<th>3 mM</th>
<th>10 mM</th>
<th>30 mM</th>
<th>Copy no.</th>
<th>Ca(^{2+}) conc. in the culture media for normal growth</th>
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<td></td>
<td></td>
<td>% total plants</td>
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<td></td>
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<td>15</td>
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<td>90.0</td>
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<td>3.492</td>
<td>8.037</td>
<td>19.380</td>
<td>2 or 3</td>
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<td>3.958</td>
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<td>3.844</td>
<td>7.709</td>
<td>18.104</td>
<td>1 or 2</td>
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<td>5.785</td>
<td>12.878</td>
<td>1</td>
<td>20</td>
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<td>0.0</td>
<td>3.888</td>
<td>3.713</td>
<td>23.527</td>
<td>1 or 2</td>
<td>10</td>
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<td>25.497</td>
<td>1</td>
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<td>0.0</td>
<td>3.792</td>
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<td>22.267</td>
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<td>15.908</td>
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<td>100.0</td>
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<td>10</td>
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<td>62.5</td>
<td>14.3</td>
<td>4.191</td>
<td>9.739</td>
<td>23.984</td>
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<td>0.0</td>
<td>3.513</td>
<td>8.188</td>
<td>18.742</td>
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<td>0.0</td>
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<td>0.0</td>
<td>5.876</td>
<td>10.896</td>
<td>22.194</td>
<td>1 or 2</td>
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<td>0.0</td>
<td>4.839</td>
<td>9.037</td>
<td>22.371</td>
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<td>50.0</td>
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<td>4.018</td>
<td>16.706</td>
<td>18.853</td>
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</table>

| Russet Norkotah lines | CAX1 #34 | 0.0 | 0.0 | 0.0 | 4.190 | 7.736 | 11.403| 0        | 3  |
| CAX1 #36 K-3-1        | CAX1 #36 | 75.0 | 25.0 | 0.0 | 5.210 | 11.745| 19.863| 1        | 20 |
| CAX1 #36 K-3-2        | CAX1 #36 | 100.0| 50.0 | 25.0| 4.041 | 8.066 | 28.559| 1        | 15 |
| CAX1 #49¶             | CAX1 #49 | 12.5 | 0.0 | 0.0 | 5.592 | 7.262 | 24.712| 2        | 10 |

† The line AT1_02_01_01 was renamed Line 1 for data presented in Table 2 and the figures.
‡ The line AT1_08_02_01 was renamed Line 2 for data presented in Table 2 and the figures.
§ The line AT2_01_09_01 was renamed Line 3 for data presented in Table 2 and the figures.
¶ The line CAX1 #49 showed short internodes and malformed leaves.
The presence or absence of CaC\textsubscript{2}O\textsubscript{4} crystals in leaves and stem tissue was visualized using polarized light microscopy (Fig. 3C–3F). Calcium oxalate crystals were detected in the mesophyll of the leaves and vascular tissue of \textit{sCAX1} (Fig. 3C and 3E). The crystals were seen also in the epidermal cells and trichomes (data not shown). Moreover, these crystals were observed at both the 1 and 10 mM Ca\textsuperscript{2+} treatments. At 10 mM, the crystals were more abundant (Fig. 3C–3F shows crystals observed at the 10 mM Ca\textsuperscript{2+} treatment). These crystals were not isolated for identification, but we strongly believe they are most likely CaC\textsubscript{2}O\textsubscript{4} crystals because they have been indicated to be present in most plant species and part of a mechanism for regulating Ca\textsuperscript{2+} in plants (Franceschi and Nakata, 2005).

Detection of Internal Tuber Defects in \textit{sCAX1}-Overexpressing Lines

The incidence of tuber internal defects, between \textit{sCAX1} lines and their wild types under greenhouse conditions, indicated that the internal defect called hollow heart, a central cavity in the tuber, was more frequent in the \textit{sCAX1} lines (Fig. 4). Atlantic transgenic lines had a probability of 0.40 for the occurrence of hollow heart compared to 0.02 in the wild type at 1 mM Ca\textsuperscript{2+} (Fig. 5B). Similar results were observed in Russet Norkotah transgenic lines.

Effects of the Overexpression of \textit{sCAX1} on Total Calcium in the Plant and Tubers

The effect of the overexpression of \textit{sCAX1} on total Ca\textsuperscript{2+} was tested on the shoot above media using four whole plants grown under in vitro conditions to allow for more precise control of nutrition. Compared with the wild type, total Ca\textsuperscript{2+} was slightly higher in the \textit{sCAX1} lines at some Ca\textsuperscript{2+} concentrations (Table 1).

Comparing the total tuber Ca\textsuperscript{2+} content of wild-type lines vs. \textit{sCAX1} lines, significant differences were detected for mean total Ca\textsuperscript{2+} in \textit{sCAX1} lines; a pairwise comparison also shows which \textit{sCAX1} lines are significantly different to their respective wild type (Fig. 6A).

In addition, cell wall content extracted from tuber fresh tissue was recorded as the percentage of cell walls in 100 g of fresh tuber tissue. The results showed that for the same amount of fresh tuber tissue, the Atlantic and Russet Norkotah \textit{sCAX1} lines had a significantly reduced amount of cell walls compared with the wild type (Fig. 6B).

The Effect of Calcium on Physiological Defects in Plants Overexpressing \textit{sCAX1}

Growing at Ca\textsuperscript{2+} concentrations above normal, the probabilities of having shoot tip damage and internal defects were drastically reduced (in other words, the probability of healthy, normal-looking plants was increased) for plants grown at >10 mM Ca\textsuperscript{2+} concentrations (Fig. 5A and 5B).

<table>
<thead>
<tr>
<th>Line</th>
<th>Difference between ( CAX1 ) and ( EF1-\alpha ) (( \Delta Ct = Ct_{\text{EF1-\alpha}} - Ct_{\text{CAX1}} ))</th>
<th>Relative expression between ( CAX1 ) and ( EF1-\alpha ) (( 2^{\Delta Ct} ))</th>
<th>Relative expression between lines (( 2^{\Delta Ct} ))</th>
</tr>
</thead>
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<tr>
<td>Atlantic wild type</td>
<td>28.36</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Line 1</td>
<td>5.38</td>
<td>0.22</td>
<td>1.2</td>
</tr>
<tr>
<td>Line 2</td>
<td>5.60</td>
<td>0.021</td>
<td>1.0</td>
</tr>
<tr>
<td>Line 3</td>
<td>4.63</td>
<td>0.040</td>
<td>2.0</td>
</tr>
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</table>

Table 2. Analysis of \textit{sCAX1} expression in relation to the housekeeping gene \textit{EF1-\alpha} in three \textit{sCAX1} Atlantic lines. Expression was evaluated using real-time polymerase chain reaction with the Evagreen dye using the CFX96 Instrument.
Plant height and fresh weight were evaluated under in vitro conditions as indicators of plant health. Increasing Ca\(^{2+}\) concentration in the media or the nutrient solution had a positive effect on the overall plant health of all sCAX1 lines. Evaluating in vitro plants for the effect of different Ca\(^{2+}\) concentrations in the culture media, significant differences in height and fresh weight between the wild type and all sCAX1 lines were observed at normal, 3 mM Ca\(^{2+}\) concentration. At higher Ca\(^{2+}\) concentrations, differences compared with the wild type were significant only for Line 3 at 10, 15, and 20 mM. An overall reduction of differences in plant height and fresh weight between wild-type and sCAX1 lines was observed at higher Ca\(^{2+}\) concentrations (Fig. 5C and 5D).

Indeed, the probability of shoot tip damage measured on in vitro plants was significantly reduced for sCAX1 lines grown at higher Ca\(^{2+}\) concentrations in the culture media but still 10 to 30 times higher than the wild type (Fig. 5A).

Similarly, the evaluation of tubers harvested from plants grown at different Ca\(^{2+}\) concentrations in the nutrient solution indicated that the probability of hollow heart was significantly reduced for sCAX1 lines at higher Ca\(^{2+}\) concentrations. In fact, at a Ca\(^{2+}\) concentration of 15 mM, the three sCAX1 lines showed similar incidence of hollow heart compared with the wild type (Fig. 5B).

**DISCUSSION**

**Overexpression of sCAX1 Can Lead to Calcium Sequestration in the Vacuole**

The expression of sCAX1 in potato causes an increased transport of Ca\(^{2+}\) into the vacuole, but what happens to that extra Ca\(^{2+}\) in the vacuole and how is it stored? We
know from previous reports that $\text{Ca}^{2+}$ is hardly redistributed to other parts of the cell once it is stored in the vacuole (Gilliham et al., 2011). Therefore, $\text{Ca}^{2+}$ stored in the vacuole does not mobilize and might be forming salts with other compounds. One type of $\text{Ca}^{2+}$ salts stored in vacuoles is $\text{CaC}_2\text{O}_4$. Calcium oxalate is abundant in certain plant species such as $\text{Oxalis}$, beets ($\text{Beta vulgaris}$ L.), and spinach ($\text{Spinacia oleracea}$ L.) and has been reported as a major regulator of $\text{Ca}^{2+}$ in plant cells (Franceschi and Nakata, 2005).

In our study, we propose that the extra $\text{Ca}^{2+}$ transported to the vacuole due to the overexpression of $\text{sCAX1}$ is sequestered in the form of $\text{CaC}_2\text{O}_4$ in different tissues of leaves (Fig. 3). The presence of $\text{CaC}_2\text{O}_4$ in tubers was not tested, but it is most likely that the extra $\text{Ca}^{2+}$ in tuber vacuoles is also stored in the form of $\text{CaC}_2\text{O}_4$.

Calcium oxalate forms crystals that vary in bioavailability, depending on their hydration, purity, and size (Libert and Franceschi, 1987). A study of bioavailability and absorption of $\text{Ca}^{2+}$ showed a reduced incorporation into bones of mice and 10% reduction in absorption of $\text{Ca}^{2+}$ from $\text{sCAX1}$-expressing carrots, even though the total concentration of $\text{Ca}^{2+}$ in 100 g of transgenic carrots was 42% higher compared with an equal quantity of the control, indicating that not all of the $\text{Ca}^{2+}$ sequestered in the vacuole by ectopic expression of $\text{sCAX1}$ is bioavailable (Morris et al., 2008). Therefore, efforts to improve the nutritional quality of potatoes using the expression of $\text{sCAX1}$ should evaluate $\text{Ca}^{2+}$ bioavailability and absorption to determine to what extent $\text{CaC}_2\text{O}_4$ in potatoes could contribute to improve human nutrition.

Fig. 5. Various traits evaluated in Atlantic wild-type and $\text{sCAX1}$ lines at different $\text{Ca}^{2+}$ concentrations on in vitro plants and greenhouse-grown tubers. (A) Probability of shoot tip damage in relation to media concentration. (B) Probability of hollow heart in the tubers of greenhouse grown plants at various $\text{Ca}^{2+}$ concentrations. (C) Plant height in relation to media $\text{Ca}^{2+}$ concentration. (D) Plant fresh weight in relation to media $\text{Ca}^{2+}$ concentration. Experimental design was complete randomized block design with two to four replicates (plants). Significance of pair-wise mean comparison with the wild type is also indicated. Significance: (.) $P < 0.1$, (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. 
Overexpression of sCAX1 Caused Calcium Deficiency Symptoms in Shoot Tips, Leaves, and Internal Physiological Defects in Tubers

Our results indicate that the overexpression of sCAX1 causes Ca\(^{2+}\) deficiency symptoms observed in in vitro and greenhouse-grown plants of potato (Fig. 2 and 4). Potato plants grown under very low amounts of Ca\(^{2+}\) such as 30 µM are known to show deficiency symptoms such as shoot tip damage, leaf margin and tip necrosis, and leaf lamina rolled towards the midrib (Singh and Sharma, 1972; Seling et al., 2000; Busse et al., 2008). All these symptoms were observed in the sCAX1 transgenic lines at normal Ca\(^{2+}\) levels. Furthermore, shoot tip necrosis and hollow heart were mitigated as the media Ca\(^{2+}\) concentration increased in these transgenic lines (Fig. 5A and 5B). Ours is the first study to document the development of classic Ca\(^{2+}\) deficiency symptoms in potato sCAX1 plants.

Increased transport of Ca\(^{2+}\) towards the vacuole reduces the availability of Ca\(^{2+}\) in other cell compartments, and therefore Ca\(^{2+}\) deficiency symptoms are observed in the transgenic lines expressing sCAX1 even when growing in media or soil with normal Ca\(^{2+}\) concentrations for the wild-type plants. Calcium deficiency-like symptoms have been reported in transgenic lines of tobacco that are overexpressing CAX1 (Hirschi, 1999). In tomatoes expressing sCAX1, severe alterations in plant development and morphology including increased incidence of blossom-end rot had been reported by Park et al. (2005b); however, this study did not relate this phenotype to deficiency symptoms. A posterior study in tomatoes overexpressing sCAX1 reported severe deficiency symptoms and high incidence of blossom end rot in tomato fruits (de Freitas et al., 2011).

Calcium content in potato varies in different parts of the plant: potato shoot tips are in the range of 5 mg g\(^{-1}\) dry weight, leaves are more variable but fluctuate in a range between 10 and 40 mg g\(^{-1}\) dry weight, and roots have 3 mg g\(^{-1}\) dry weight (Seling et al., 2000). Potato tubers are normally low in Ca\(^{2+}\) (Wiersum, 1966; Davies and Millard, 1985; Kratzke and Palta, 1986), in the range between 100 and 350 mg g\(^{-1}\) dry weight, depending on the cultivar (Karlsson et al., 2006). Organs with high transpiration rates accumulate more Ca\(^{2+}\), and low-transpiring organs (such as tubers) can suffer Ca\(^{2+}\) deficiencies (Gilliham et al., 2011). In plant tissues growing under Ca\(^{2+}\) deficiency, the nuclear envelope, plasma membrane, and cell walls disintegrate and tissues collapse resulting in necrosis (Marinos, 1962; Kirkby and Pilbeam, 1984). In our study, the Atlantic and Russet Norkotah sCAX1 lines showed significantly higher incidence of tuber internal defects in plants grown with normal Ca\(^{2+}\) levels, 1 mM Ca\(^{2+}\) (Fig. 4 and 6B). These results suggest that the increased transport of Ca\(^{2+}\) into the vacuoles in the sCAX1 transgenic lines has a negative effect on cell wall content and tuber quality, even though the tuber total Ca\(^{2+}\) was higher among the transgenic sCAX1 lines compared with the wild type (Fig. 4 and 5). A subcellular localization analysis found that very little Ca\(^{2+}\) is present in the cell walls (Opara and Davies, 1988). Therefore, when Ca\(^{2+}\) homeostasis is altered, cell walls can be affected, and internal defects and other deficiency symptoms may appear.
Calcium deficiency symptoms related to the overexpression of \textit{CAXI} have not been previously reported in potato. Park et al. (2005a) transformed the potato cultivar Russet Norkotah with \textit{sCAXI}. This study found that increased levels of \textit{CAXI} do not appear to alter potato growth and development, nor tuber morphology or yield, but reported some weak necrotic lesions observed on the primary transformants that were not described. Their results differ from our results. Park et al. (2005a) may not have detected \textit{Ca}^{2+} deficiency symptoms because they grew the Russet Norkotah \textit{sCAXI} lines in Metro-Mix 700, a soil mix that is amended with \textit{Ca}^{2+}. Pot mixes usually use high amounts of \textit{Ca}^{2+} to counteract the acidity of peat moss and because there are no toxicity problems associated with \textit{Ca}^{2+}. Therefore, \textit{Ca}^{2+} concentration in their pot mix may have been much higher than the normal amount needed.

**Calcium Supplementation Improves Deficiency Symptoms in Apical Shoot Tips, Leaves, and Tuber Internal Defects**

In our study, \textit{Ca}^{2+} deficiency symptoms in foliage and tubers were reduced in high \textit{Ca}^{2+} treatments (Fig. 5A). There was a variable degree of alleviation of \textit{Ca}^{2+} deficiency symptoms in the leaves of plants given the high \textit{Ca}^{2+} treatment under greenhouse conditions. Nevertheless, the \textit{Ca}^{2+} deficiency symptoms caused by the expression of \textit{sCAXI} were not completely or uniformly alleviated under greenhouse conditions, especially leaf margin necrosis. Besides the overexpression of \textit{sCAXI}, the position and evapotranspiration rate of each particular leaf likely influenced \textit{Ca}^{2+} deficiency symptoms. Under in vitro conditions, the positive effects of increased amounts of calcium on shoot development were clear, and shoot tip damage, a symptom of \textit{Ca}^{2+} deficiency, was mitigated when the MS media contained $>15$ mM \textit{Ca}^{2+}.

In our study, we observed that internal defects of transgenic plants in tubers were mitigated by supplying high \textit{Ca}^{2+} nutrient solutions containing $10$ mM \textit{Ca}^{2+} or more (Fig. 5B). The transport of \textit{Ca}^{2+} is mainly through the apoplast. Our observations indicated that tuber defects were alleviated when plants were treated with high \textit{Ca}^{2+} nutrient solutions. These results further confirm previous studies that have shown that tuber internal defects can be mitigated by supplemental application of \textit{Ca}^{2+} (Tzeng et al., 1986; Olsen et al., 1996; Palta, 1996; Kleinhenz et al., 1999; Karlsson et al., 2006; Ozgen et al., 2006).

A recent study demonstrated that some \textit{Ca}^{2+} deficiency symptoms in maize (\textit{Zea mays} L.) can be relieved by the expression of a gene encoding for the \textit{Ca}^{2+} binding protein calreticulin (Wu et al., 2012), a gene that might be involved in symplastic \textit{Ca}^{2+} mobilization. This experiment was done in tobacco and tomato where the co-expression of calreticulin, a chaperone found in the endoplasmic reticulum, in the \textit{sCAXI}-expressing lines relieved the \textit{Ca}^{2+} deficiency symptoms caused by \textit{sCAXI} (Wu et al., 2012). Therefore, we may hypothesize that if Atlantic and Russet Norkotah \textit{sCAXI} lines were co-transformed with calreticulin, \textit{Ca}^{2+} deficiency symptoms might be reduced and total \textit{Ca}^{2+} increased; however, the success of this experiment would depend on a tissue and time-specific promoters used for the expression of these genes.

Once \textit{Ca}^{2+} is deposited in the vacuole, it is rarely redistributed (Gilliham et al., 2011). Therefore, we hypothesize that \textit{Ca}^{2+} deficiency symptoms can be alleviated by: (i) increasing apoplastic \textit{Ca}^{2+} by using a higher \textit{Ca}^{2+} concentration in the solution, and (ii) generating symplastic \textit{Ca}^{2+} mobilization, or a combination of both strategies.

**Cell Wall Content in Tubers Decreases as a Consequence of Overexpressing \textit{CAXI}**

More \textit{Ca}^{2+} is being transported into the vacuole in the transgenic plants; therefore, there might be less \textit{Ca}^{2+} available in other cellular compartments, especially in the apoplast, given that \textit{Ca}^{2+} transport into the vacuole modulates apoplastic \textit{Ca}^{2+} concentrations (Conn et al., 2011). A close relationship between apoplastic \textit{Ca}^{2+} and cell wall \textit{Ca}^{2+} has been documented by previous research (Demarty et al., 1984; Pechanova et al., 2010; Gilliham et al., 2011; Wang et al., 2013). This suggests that \textit{sCAXI} transgenic lines may be affected by a decrease in apoplastic \textit{Ca}^{2+}. This reduced apoplastic calcium may compromise cell walls by decreasing the cell wall-bound \textit{Ca}^{2+} or by a reduction of the cell wall biomass. Our experiments measured cell wall biomass in tubers and found that there was $\approx 1$ g less dry weight of cell walls per 100 g of fresh tuber tissue in Atlantic and Russet Norkotah \textit{sCAXI} transgenic lines than in the control (Fig. 6B). Furthermore, this decrease in cell wall may explain shoot tip necrosis symptoms observed in the transgenic lines in our study. Weakness in the walls of expanding subapical cells has been observed to be related to \textit{Ca}^{2+} deficiency and shoot tip necrosis (Busse et al., 2008). The fact that these symptoms were alleviated at higher media \textit{Ca}^{2+} concentrations also suggests that when sufficient \textit{Ca}^{2+} is available, the cell wall integrity is maintained. It is also interesting to note that hollow heart in potato is known to be caused by an abrupt increase in growth of the tuber tissue after a dry period (Palta, 2010). This abrupt increase in growth causes tension in the center part of the tuber due to cell expansion in different directions. A \textit{Ca}^{2+}-deficient tuber with compromised cell wall integrity will start to separate during this expansion resulting in a cavity. A dramatic increase in hollow heart symptoms in the transgenic lines further supports that this serious physiological disorder of potato is related to lack of sufficient cell wall \textit{Ca}^{2+} in the tuber tissue.

The role of the H$^+/\textit{Ca}^{2+}$ antiporter \textit{CAXI} as regulator of \textit{Ca}^{2+} has been confirmed in our study. The results presented in our study show that the overexpression of \textit{sCAXI} leads
to symptoms of Ca\(^{2+}\) deficiency in leaves, compromises tissue health, and increases the probability of having internal defects in tubers. Furthermore, our study confirms that supplementing Ca\(^{2+}\) concentration in the nutrient media reduces the probability of having Ca\(^{2+}\) deficiency symptoms such as shoot tip damage and hollow heart. We suggest that these deficiency symptoms result from transport of Ca\(^{2+}\) to the vacuole, where it is sequestered and made unavailable in the form of Ca\(_2\)C\(_2\)O\(_4\). We further suggest that CAX1 is an important regulator of cell wall strength. The effects of the overexpression of sCAX1 in the Atlantic lines were the same observed in the Russet Norkotah lines overexpressing sCAX1 developed by Park et al. (2005a). Our results are in agreement with reports in other crops showing that overexpressing sCAX1 causes Ca\(^{2+}\) deficiency symptoms (de Freitas et al., 2011; Wu et al., 2012).

**Conflict of Interest**
The authors declare that there is no conflict of interest.

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**References**


edence of internal brown spot development upon calcium fer-


