Mutations in *CAX1* produce phenotypes characteristic of plants tolerant to serpentine soils

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Summary

- Plant tolerance of serpentine soils is potentially an excellent model for studying the genetics of adaptive variation in natural populations.
- A large-scale viability screen of *Arabidopsis thaliana* mutants on a defined nutrient solution with a low Ca^{2+} : Mg^{2+} ratio (1 : 24 mol : mol), typical of serpentine soils, yielded survivors with null alleles of the tonoplast calcium-proton antiporter CAX1.
- *cax1* mutants have most of the phenotypes associated with tolerance to serpentine soils, including survival in solutions with a low Ca^{2+} : Mg^{2+} ratio; requirement for a high concentration of Mg^{2+} for maximum growth; reduced leaf tissue concentration of Mg^{2+}; and poor growth performance on 'normal' levels of Ca^{2+} and Mg^{2+}.
- A physiological model is proposed to explain how loss-of-function *cax1* mutations could produce all these phenotypes characteristic of plants adapted to serpentine soils, why 'normal' plants are unable to survive on serpentine soil, and why serpentine-adapted plants are unable to compete on 'normal' soils.

Key words: adaptation, *Arabidopsis thaliana*, calcium homeostasis, CAX1, edaphic, magnesium homeostasis, mineral nutrition, serpentine soil.

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Introduction

Serpentine soils, because of their unique chemical composition, harbor striking examples of adaptive evolution in plants. Reciprocal transplant experiments between serpentine populations (or species) and their spatially adjacent populations (or sister species) growing on 'normal' soils demonstrate unequivocally that adaptation to serpentine soils has a genetic basis (Kruckeberg, 1950, 1954; Nyberg Berglund *et al.*, 2003; Rajakaruna *et al.*, 2003). Serpentine soils and normal soils are frequently found in a mosaic pattern with sharp boundaries between the two soil types. Adaptation to serpentine soil can occur over short distances, often in the face of considerable gene exchange between serpentine-adapted and normal populations, presumably because natural selection for serpentine tolerance is strong. This very local scale of adaptive evolution makes serpentine-adapted plant populations useful systems for discovering the genetic basis of traits that contribute to ecological specialization (Pepper & Norwood, 2001).

Although the 'serpentine syndrome' (Jenny, 1980) requires adaptation to a variety of physical and chemical factors in the soil – low moisture-holding capacity, low availability of nitrogen and phosphorus, and often high concentrations of heavy metals such as nickel or chromium – Kruckeberg (1985) suggests that plant adaptation to serpentine soils is largely driven by the evolution of physiological tolerance to two universal characteristics of serpentine: very low concentrations of calcium ions, and very high concentrations of magnesium ions. Serpentine soils nearly always have a Ca^{2+} : Mg^{2+} molar ratio of less than unity (often < 1 : 10, and sometimes < 1 : 100), while normal soils have a Ca^{2+} : Mg^{2+} ratio > 1 : 1 (Epstein, 1972). [For comparison, Hoagland's nutrient solution has a Ca^{2+} : Mg^{2+} ratio of 4 : 1 (Epstein, 1972).] Because a great deal is known about the physiology and biochemistry of Ca^{2+} (Felle, 1991; Rudd & Franklin-Tong, 1999; Miedema *et al.*, 2001; Sanders *et al.*, 2002) and Mg^{2+} (Li *et al.*, 2001) in plants, and many of the genes involved in Ca^{2+} and Mg^{2+} homeostasis have been identified (Hirschi, 2001; Li *et al.*, 2001; Cheng *et al.*, 2003; Cheng *et al.*, 2004), it may now be possible to discover and describe, at the molecular level, variation in a physiological phenotype with clear ecological and evolutionary significance in natural plant populations.

To identify candidate genes likely to be involved in adaptation to serpentine soils, I used a chemically defined nutrient solution that mirrors the low Ca^{2+} : Mg^{2+} ratio (1 : 24) of authentic serpentine soils to perform a viability screen on a large collection of activation-tagged mutants (Weigel *et al.*, 2000) in *Arabidopsis thaliana.*

Materials and Methods

Nutrient solutions

Chemically defined nutrient solutions (pH = 5.5) were prepared as described (Epstein, 1972), except that Miller's Iron Chelate DP (Miller Chemical and Fertilizer Corp., Hanover, PA, USA) was used instead of Fe-EDTA. All nutrient solutions were prepared with deionized water and contained magnesium sulfate (1 mmol l^{-1}), ammonium phosphate (2 mmol l^{-1}), potassium nitrate (6 mmol l^{-1}), Miller's Iron Chelate DP (20 mg l^{-1}), potassium chloride (50 µmol l^{-1}), boric acid (25 µmol l^{-1}), manganese chloride (2 µmol l^{-1}), zinc chloride (2 µmol l^{-1}), cupric chloride (500 nmol l^{-1}) and sodium molybdate (500 nmol l^{-1}). To keep nitrate concentration and osmotic strength constant, while varying the concentrations of calcium and magnesium ions in nutrient solutions B–F, calcium nitrate and magnesium nitrate were mixed in different proportions that sum to 4 mmol l^{-1} (Table 1). Solution B (Table 1) is Hoagland's nutrient solution, a standard defined growth medium for plants (Epstein, 1972). Solution F is designated 'faux serpentine'.

Glasshouse growth of *Arabidopsis thaliana* on nutrient solutions B–F

Wild-type seed of *Arabidopsis thaliana* accession Wassilewskija (Ws) was obtained from Rick Amasino (University of Wisconsin). For each of five nutrient solutions (B, C, D, E or F), seeds were sown onto rock wool cubes (4 × 4 × 4 cm; Grodan A/S, Denmark) saturated with nutrient solution. After germination the seedlings were thinned to one per cube. Plants were irrigated to saturation daily with the appropriate nutrient solution. The glasshouse was maintained at 20°C during the day and 15°C at night. Natural light was supplemented with sodium vapor lamps on a 16 h d^{-1} photoperiod. At 4 wk after sowing, the above-ground portion of the plant was harvested, dried for 24–36 h at 60°C, and weighed to the nearest 0.1 mg.

'Faux serpentine-tolerant' mutant screen

An activation-tagged (pSKI015; Weigel *et al.*, 2000) population of *Arabidopsis thaliana* (Ws) was obtained from Rick Amasino (University of Wisconsin). Approximately 920 000 seeds from pools 1–230 (≈10 seeds from each of ≈92 000 independently tagged lines) were sown onto rock wool bats (7 cm thick) at a density of 30 seeds cm^{-2}. The rock wool was saturated with solution F (Table 1). A polyethylene tent was used for the first 5 d to maintain high humidity for germination. The rock wool was watered to saturation with solution F every day after the tent was removed. Glasshouse growing conditions were as described above.

At 12 d after sowing, surviving seedlings ($n = 30$) were excised from the rock wool and transplanted to Sunshine #4 growing medium (Sun Gro Horticulture, Seba Beach, AB, Canada), where they received a complete fertilizer solution (Peters Peat Lite, Scotts Co., Marysville, OH, USA) twice weekly, with clear water on the other days. All 30 selected seedlings survived to flower, and all but one were self-fertile. Selfed seeds were collected from each of the putative 'faux serpentine-tolerant' (FST) lines (numbers FST-1 to FST-30, with line FST-29 missing because of self-sterility).

Tests for presence of the FST phenotype were performed by sowing seeds on rock wool cubes saturated with solution F (Table 1). Seedlings surviving to produce a pair of true leaves were scored as FST; wild-type seedlings bleach and die at the cotyledon stage. Tests for the presence of the *BAR* gene in the activation tag (Weigel *et al.*, 2000) were carried out by spraying seedlings twice weekly with a 1 : 1000 dilution of Finale (Farnam Co. Inc., Phoenix, AZ, USA) having a final concentration of 58 ppm glufosinate ammonium.

Inverse PCR of the activation tag and flanking integration site

For each FST line, leaf tissue from 10 plants grown in Sunshine #4 was pooled for DNA extraction. Genomic DNA

Table 1 Sources of calcium and magnesium ions in nutrient solutions B–F

Solution	$Ca(NO_3)_2$ (mmol l^{-1})	$Mg(NO_3)_2$ (mmol l^{-1})	$MgSO_4$ (mmol l^{-1})	Ca^{2+} : Mg^{2+} (mol : mol)	Ca^{2+} : Mg^{2+} (ppm : ppm)
B	4.0	0.0	1.0	4.00	6.60
C	2.0	2.0	1.0	0.67	1.10
D	1.0	3.0	1.0	0.25	0.41
E	0.5	3.5	1.0	0.11	0.18
F	0.2	3.8	1.0	0.04	0.07

Table 2 Genetic markers used for positional cloning of the gene responsible for the 'faux serpentine-tolerant' phenotype in *Arabidopsis thaliana*

Locus	Forward primer 5′→3′	Reverse primer 5′→3′	PCR annealing temperature (°C)	Polymorphism
At2g31990 (WT)	GATTTCCGGCGACATTTACG	GAAGTTGGTTCCCCATGCTG	55	
At2g31990::pSKI015	GATTTCCGGCGACATTTACG	GATGTGATATCTAGATCCGAAACTATC	55	
MSAT2.4	TGGGTTTTTGTGGGTC	GTATTATTGTGCTGCCTTTT	50	length
At2g35060	CAAGAGTAGAAGCAGCAACGAT	AACAACAACACCTGTAATAGTA	55	*Afl*III RFLP
At2g38940	ATGGCAAGGGAACAATTACAAGT	AGTGAAGAGGATACCTAAGAAGT	55	*Mnl*I RFLP

was extracted using the FastPrep system (MP Biomedicals, Irvine, CA, USA), digested with *Mse*I (New England Biolabs, Beverly, MA, USA), heated to 65°C for 5 min to inactivate the restriction enzyme, diluted to a DNA concentration of ≈1 ng μl^{-1}, and circularized with T4 DNA ligase (New England Biolabs).

Primers for inverse PCR (iPCR) of the activation tag integration site into the *Arabidopsis* genome were designed within the cauliflower mosaic virus (CaMV) 35S enhancer at the right border of the activation tag T-DNA (Weigel *et al*., 2000). The forward (rightward, from the CaMV 35S enhancer into the flanking regions) primer is 5′-ACGACACTCTCGTC-TACTCC-3′ and the reverse (leftward) primer is 5′-CGTCGTTTTACAACGTCGTG-3′. iPCR reactions were carried out in 10 μl 10 mmol l^{-1} Tris–Cl pH 8.3, 50 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 1.3 mg ml^{-1} bovine serum albumin, 200 μmol l^{-1} of each dNTP, 5 μmol l^{-1} of each primer, 0.2 U AmpliTaq (Roche Molecular Systems, Branchburg, NJ, USA), and ≈1 ng circularized *Arabidopsis* genomic DNA template prepared as described above. Thermocycling conditions were 30 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s. iPCR products were resolved on ethidium-stained 1.5% agarose gels and examined visually to organize (provisionally) the FST lines into complementation groups. iPCR products were cloned into pGEM-T Easy (Promega, Madison, WI, USA), transformed into *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, CA, USA), plasmid DNA prepared with QIAprep (Qiagen, Valencia, CA, USA), and sequenced with BigDye chemistry (Applied Biosystems, Foster City, CA, USA).

Positional cloning

PCR primers used to amplify genetic markers segregating in the F_2 generation of a cross between line FST-20 (Ws background) and Columbia *erecta* (Col-*er*) are shown in Table 2. Microsatellite marker MSAT2.4 was developed by Bell & Ecker (1994). The other markers were developed as part of the project described in this paper. PCR was carried out as described above, except that annealing temperature was optimized for each primer pair (Table 2).

Results

Growth of *Arabidopsis* (Ws) on nutrient solutions with varying Ca : Mg ratios

When grown in a series of nutrient solutions with decreasing Ca^{2+} : Mg^{2+} ratios (Table 1), wild-type *A. thaliana* (Ws) behaves as a typical plant which is not adapted to serpentine soils (Walker *et al*., 1955). Dry biomass (mean ± SEM) at flowering was not significantly different (two-tailed *t*-test, experiment-wise $\alpha \leq 0.05$, Bonferroni corrected) for *Arabidopsis* grown in solutions B (28.1 ± 12.8 mg, $N = 17$); C (32.7 ± 15.1 mg, $N = 15$); or D (33.4 ± 20.5 mg, $N = 12$). However, biomass was reduced significantly ($P \leq 0.01$) to 8.5 ± 2.7 mg ($N = 2$) on solution E.

No seedlings survived to flowering on solution F. All seedlings bleached and died at the cotyledon stage.

The growth of wild-type *Arabidopsis* Ws is thus reduced markedly as the Ca^{2+} : Mg^{2+} molar ratio declines from 0.25 (solution D) to 0.11 (solution E). A further decrement to 0.04 (solution F) is lethal.

Discovery and initial genetic analysis of FST mutants

With the survival threshold value for Ca^{2+} : Mg^{2+} molar ratio determined to lie between 0.11 and 0.04, I screened a large collection of activation-tagged *Arabidopsis* mutants for their ability to survive and grow on solution F, which is lethal to wild-type Ws. Within 10 d of sowing the mutagenized seeds, FST mutants were easily distinguished among the dead and dying wild-type seedlings (Fig. 1).

The FST seedlings were rescued by transplanting to a standard glasshouse soil mix (Sunshine #4), grown to flowering, and allowed to produce selfed seed. Each of the resulting 29 independent FST lines was found to contain at least one activation tag derived from the T-DNA in pSKI015 (Weigel *et al*., 2000), as judged by their ability to produce seedlings resistant to glufosinate.

The FST lines were clustered into provisional complementation groups by iPCR. The largest complementation group contained 13 of the 29 lines, and was selected for further

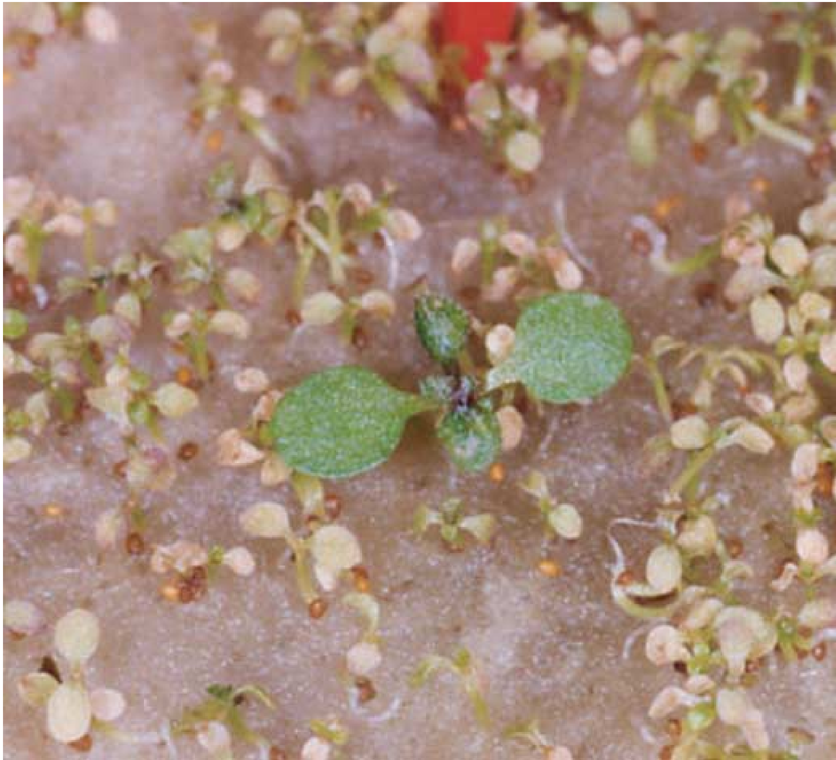

Fig. 1 A 'faux serpentine-tolerant' mutant growing on solution F (Ca^{2+} : Mg^{2+} = 0.04 mol : mol). Magnification ≈ 4×.

investigation. Three of the 13 lines in this complementation group appeared to be descended from a mutant heterozygous for a single activation tag, based on the pattern of segregation (≈3 : 1 resistant : sensitive) observed in the progeny test for glufosinate resistance. One of these segregating lines, FST-20, became the focus of detailed genetic analysis for the FST trait.

Identification and genetic analysis of the activation-tagged allele in line FST-20

Flanking DNA sequence from the FST-20 iPCR product suggested that the activation-tagging T-DNA was inserted into the single intron of the gene encoding At2g31990, a member of the exostosin protein family at ≈13.5 Mbp on chromosome II. This insertion site was confirmed by PCR analysis. PCR primers were designed to distinguish the wild-type intron from the mutant allele carrying the activation tag inserted from pSKI015. Both wild-type and mutant alleles share a forward primer in the intron, with a reverse primer from either the intron (wild-type allele) or activation tag (mutant allele At2g31990::pSKI015) (Table 2). These primers were used to identify segregants in line FST-20 that either are homozygous for the wild-type allele or homozygous for the mutant allele of At2g31990. Homozygous plants were allowed to produce selfed seed, yielding pure lines of each allele. In addition, several homozygous mutant plants were crossed to Ws to produce known heterozygotes to test for dominance of the FST phenotype derived from line FST-20.

Activation tags are capable of generating both dominant (gain-of-function) and recessive (loss-of-function) mutations (Weigel *et al.*, 2000). Based on the presence of the FST phenotype in the heterozygous founder of line FST-20, I expected that the FST phenotype was produced by a dominant allele at (or very near) At2g31990. Surprisingly, I found instead that the F_1 seedlings resulting from a cross between homozygous mutant plants and wild-type Ws failed to show the FST phenotype when tested on solution F. Furthermore, pure lines descended from FST-20, identified as homozygous for the wild-type allele at At2g31990, unexpectedly showed the FST phenotype on solution F. The pure lines homozygous for the mutant allele also had the FST phenotype, as expected. Together these findings suggest that the *fst-20* mutation is recessive, and that the activation-tagged gene At2g31990 is not responsible for the FST phenotype in FST-20. As untagged mutations can represent two-thirds of the mutations in an activation-tagging experiment (McElver *et al.*, 2001), it is to be anticipated that many alleles identified in mutant screens will need to be cloned positionally.

Positional cloning of the *fst-20* allele responsible for the FST phenotype

A cross was made between a Col-*er* mutant and an FST plant (*fst-20*/*fst-20*) in the Ws background. F_1 plants were allowed to self-pollinate, producing F_2 seeds. F_2 seeds were sown on rock wool saturated with solution F to select for the FST

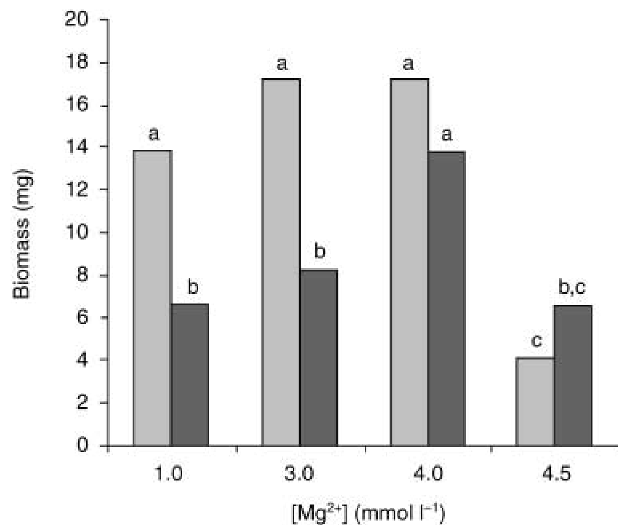

Fig. 2 Growth of wild-type *Arabidopsis thaliana* (Ws, grey bars) and a *cax1* mutant (FST-20, black bars) on nutrient solutions B–E (left to right; Table 1). Different letters above bars indicate significant differences in dry biomass (two-tailed *t*-test, experiment-wise $\alpha \leq 0.05$, Bonferroni corrected).

phenotype. As the FST phenotype is produced by a recessive allele, each surviving F_2 plant was expected to be homozygous for the *fst-20* allele.

The large fraction (10/13) of FST lines homozygous for both the mutant At2g31990 allele and the *fst-20* allele indicated that the two loci may be linked. A microsatellite marker (MSAT2.4, Bell & Ecker, 1994; this study Table 2) at 14.0 Mbp on chromosome II, near At2g31990 (13.5 Mbp), was used to test this hypothesis of linkage. Among 94 *fst-20*/*fst-20* F_2 plants, 86 were also homozygous for the Ws allele at MSAT2.4, while the other eight were heterozygous, yielding a genetic distance estimate of 4.3 cM between MSAT2.4 and *fst-20*.

With an average physical : genetic distance ratio of ≈200 kbp cM^{-1} in *A. thaliana*, a visual inspection of genes 0.6–1.0 Mbp on either side of MSAT2.4 yielded a putative potassium transporter (At2g35060) at 14.7 Mbp on chromosome II. PCR primers were designed to amplify a portion of this gene, and a restriction fragment length polymorphism between Ws and Col-*er* alleles at this locus was revealed by *Afl*III (Table 2). Among 116 *fst-20*/*fst-20* F_2 progeny (including the 94 F_2 progeny used to map MSAT2.4), eight recombinant marker genotypes were found, giving a revised estimate of the physical position of the *fst-20* allele at 16.3 Mbp on chromosome II.

A phosphate transporter (At2g38940) was found at 16.2 Mbp. RFLP (Table 2) genotypes of the 116 F_2 plants showed just one recombination event, and that event was in a plant which was nonrecombinant at MSAT2.4 and At2g35060. The *fst-20* allele was now flanked with markers, with an estimated physical location of 16.0 Mbp on chromosome II.

Null alleles of the calcium-proton antiporter CAX1 produce the FST phenotype

The *CAX1* gene at 15.9 Mbp on chromosome II is an obvious candidate for the gene which, when mutated to a loss-of-function allele, produces the FST phenotype. CAX1 is a member of a family of tonoplast calcium-proton antiporter proteins (Hirschi *et al.*, 1996; Hirschi, 2001). Complementation testing of the *fst-20* allele was carried out by crossing *fst-20*/*fst-20* plants to *cax1* mutants, and to mutants at the related *cax2* and *cax3* loci (all *cax* mutants were in a Columbia-0 genetic background; Cheng *et al.*, 2003), provided by Kendal Hirschi (Baylor University, TX, USA). Control crosses were also made between *fst-20*/*fst-20* and Col-0. F_1 seeds, along with control seeds homozygous for the relevant wild-type and mutant alleles, were sown on rock wool cubes saturated with solution F.

The *cax1* homozygous, *fst-20* homozygous, and *fst-20* × *cax1* F_1 seedlings showed the FST phenotype on solution F, while Ws, Col-0, *cax2*, *cax3*, and their crosses to *fst-20* failed to survive. All evidence indicates that the FST phenotype is produced by a knockout of CAX1 function.

Growth and mineral content phenotypes of *cax1* mutants

As observed in the initial experiments, growth of wild-type Ws plants is unaffected by Mg^{2+} concentrations of 1.0–4.0 mmol l^{-1} (Ca^{2+} : Mg^{2+} molar ratio 4.00–0.25), but growth is reduced significantly at higher Mg^{2+} (and lower Ca^{2+}) concentrations (Fig. 2). The *cax1* mutant line FST-20 shows a different response, with a peak of growth at 4.0 mmol l^{-1} Mg^{2+}, and reduced growth at higher and lower values (Fig. 2). This requirement for increased Mg^{2+} to produce maximum growth is typical of serpentine-adapted plants (Madhok, 1965; Madhok & Walker, 1969; Tyndall & Hull, 1999). It is also clear that, while the recessive *cax1* allele allows homozygous mutants to survive in very high levels of Mg^{2+} and low Ca^{2+} : Mg^{2+} ratios, as on solution F, the mutants pay a significant growth penalty when grown in the more normal concentrations of Ca^{2+} and Mg^{2+} in solutions B (4 mmol l^{-1} Ca^{2+}; 1 mmol l^{-1} Mg^{2+}) and C (2 mmol l^{-1} Ca^{2+}; 3 mmol l^{-1} Mg^{2+}) (Fig. 2).

Tissue mineral concentrations have been measured in the leaves of *cax1* mutants, grown in Hoagland's solution, as part of a large-scale genomics project in *A. thaliana* (Hirschi, 2003; Lahner *et al.*, 2003). Mineral content is reported as the number of standard deviations (σ) above or below the mean for wild-type plants grown in the same trays with the mutants. For the 12 *cax1* plants in the database (http://hort.agriculture.purdue.edu/Ionomics/database.asp; 6 November 2004), the mean leaf Mg^{2+} levels are reported to be 0.7 σ below normal, with all but one of the 12 plants having a negative value for σ (Wilcoxon matched pairs signed ranks test, $P = 0.001$). Under these growing conditions there

is no significant effect of the *cax1* mutation on tissue Ca^{2+} concentration relative to wild-type (mean = -0.2σ, $P = 0.4$).

Discussion

Loss of CAX1 function explains many features of serpentine tolerance

Tyndall & Hull (1999) list four traits frequently found in the many plant taxa that have independently adapted to serpentine soils:

1 greater tolerance of low Ca^{2+} and high Mg^{2+} concentrations;
2 higher Mg^{2+} requirement for maximum growth;
3 lower Mg^{2+} absorption; and
4 Mg^{2+} exclusion from leaves.

Remarkably, loss-of-function mutations in a single gene, *CAX1*, can produce at least three of these four traits in *A. thaliana*. First, greater tolerance of low Ca^{2+} and high Mg^{2+} concentrations is shown by the ability of *cax1* homozygotes to survive and grow on solution F, which is lethal to *A. thaliana* plants with the wild-type *CAX1* allele (Fig. 1). Tolerance of low Ca^{2+} and high Mg^{2+} levels has been described previously for *cax1* mutants of *A. thaliana* (Cheng *et al*., 2003). Second, *cax1* mutants require a higher concentration of Mg^{2+} for maximum growth (Fig. 2). Third, *cax1* mutants have significantly reduced levels of Mg^{2+} (-0.7σ) in their leaves. Although no studies have been done to measure the Mg^{2+} uptake of roots in *cax1* mutants, it is possible that *cax1* mutations can also produce the fourth trait characteristic of serpentine-tolerant plants.

Kruckeberg (1950, 1954) explains the high levels of plant endemism on serpentine soils as the result of the inability of nonserpentine plants to survive on the unique chemistry of serpentine soils, and the inability of serpentine-adapted plants to compete on normal soils. The *cax1* mutation in *A. thaliana* produces exactly the same trade-off, conferring the ability to survive on solution F while simultaneously reducing growth on solutions B and C relative to wild-type plants (Fig. 2).

A model for serpentine soil tolerance based on loss of CAX1 function

If *cax1* null mutations can produce most of the phenotypes characteristic of tolerance to the low $Ca^{2+} : Mg^{2+}$ ratio in serpentine soil, is there a plausible physiological model that provides a mechanistic explanation for these phenotypes? One such hypothesis is presented here.

For wild-type plants growing in normal soil, calcium ion concentration ($[Ca^{2+}]$) in the soil solution is in the 10^{-3} mol l^{-1} range, while cytoplasmic $[Ca^{2+}]$ must be maintained within narrow limits around 10^{-7} mol l^{-1} (Felle, 1991; Trewavas & Knight, 1994; Rudd & Franklin-Tong, 1999). When calcium channels in the plasma membrane are opened as part of a signaling cascade, the large gradient in $[Ca^{2+}]$ across the plasma membrane causes cytoplasmic $[Ca^{2+}]$ to spike upward. Following signal transduction mediated by this Ca^{2+} spike, cytoplasmic Ca^{2+} must be returned to its resting concentration. CAX1, a high-capacity calcium-proton antiporter that resides in the tonoplast, helps maintain cytoplasmic Ca^{2+} homeostasis by pumping excess calcium ions from the cytoplasm into the vacuole (Cheng *et al*., 2003).

In wild-type plants growing in serpentine soil, with very low $[Ca^{2+}]$ and very high $[Mg^{2+}]$, cytoplasmic $[Ca^{2+}]$ becomes too low as a result of the constitutive activity of CAX1 pumping Ca^{2+} from the cytoplasm into the vacuole. I propose that a nonselective cation channel (NSCC; for a recent review see Demidchik *et al*., 2002) in the plasma membrane, with a pore size sufficient to admit the large hydrated Mg ion, opens in response to the $[Ca^{2+}]$ deficit in the cytoplasm. Under normal circumstances this NSCC would primarily conduct Ca^{2+} across the plasma membrane (as Ca^{2+} is the most abundant divalent cation in normal soil solutions) and thereby restore Ca^{2+} homeostasis to the cytoplasm. However, in serpentine soil solutions the opening of this NSCC causes a large influx of Mg^{2+}. The chronically low cytoplasmic $[Ca^{2+}]$ produced by CAX1 keeps this NSCC open more-or-less continuously in a futile effort to increase cytoplasmic $[Ca^{2+}]$, and ultimately results in poisoning the plant with high cytoplasmic $[Mg^{2+}]$. Magnesium toxicity is one of the mechanisms hypothesized for the inability of most plants to survive on serpentine soils (Proctor, 1970, 1971, 1999; Brooks & Yang, 1984; Brooks, 1987). The other major hypothesis for the lethality of serpentine soils is that competition for uptake between Ca^{2+} and Mg^{2+} leads to a fatal calcium deficiency (Grover, 1960; Madhok, 1965; Madhok & Walker, 1969). Both these hypotheses are supported in the proposed CAX1 model – the proximate cause of plant death is Mg^{2+} toxicity, but ultimately that is brought about by a Ca^{2+} deficiency produced by competition between Ca^{2+} and Mg^{2+} for transit through the NSCC.

Mutant plants lacking CAX1 function are able to survive on serpentine soils because they maintain cytoplasmic $[Ca^{2+}]$ within normal limits, even though Ca^{2+} is at very low concentration in the soil solution. Without CAX1 to deplete cytoplasmic Ca^{2+}, the Mg^{2+}-conducting NSCC remains closed most of the time, preventing the accumulation of a lethal concentration of Mg^{2+} in the plant tissues. However, the closure of this NSCC causes *cax1* mutants to have a higher Mg^{2+} requirement for maximum growth, either because the proposed NSCC is not open often enough to admit sufficient Mg^{2+} when $[Mg^{2+}]$ is at normal levels, or because another Mg^{2+} transporter with lower affinity or conductance must be used for Mg^{2+} uptake in a *cax1* mutant. For the same reason, $[Mg^{2+}]$ in the leaves of *cax1* mutants is reduced in all soil solutions.

cax1 mutants grown in normal soil solutions are susceptible to Ca^{2+} toxicity because of their compromised ability to sequester excess Ca^{2+} in the vacuole (Cheng *et al*., 2003). This, along with their requirement for high $[Mg^{2+}]$, may explain the reduced growth of serpentine-adapted plants, and their consequent

inability to compete with wild-type plants, on normal soils (Kruckeberg, 1950, 1954).

Unanswered questions and future directions

The existence of the NSCC proposed in the model above is as yet undetermined. If it does exist, elucidation of the functional properties of this NSCC is critical to understanding how tolerance to serpentine soil might arise. If this NSCC is not required for uptake of some critical plant nutrients, then a knockout mutation should produce the same tolerance for high [Mg^{2+}] that is observed for the *cax1* mutants. So far, however, no FST mutants having a lesion in a gene other than *CAX1* have been discovered. It could be that the NSCC is required for plant survival, but that modulation of its opening by CAX1 function (or nonfunction, in the case of *cax1* mutants) is sufficient to produce the FST phenotype. If this is the case, then simple knockout mutant screens will not be effective in identifying the NSCC.

The most important unresolved issue is whether *cax1* mutations play a role in the adaptation of natural plant populations to serpentine soil. In principle, there are several approaches to testing this hypothesis. First, serpentine-adapted plants and their closest nonserpentine relatives could be tested for expression of *CAX1* mRNA and protein, with the expectation that at least some serpentine-adapted plant taxa will lack expression. Second, in those serpentine-tolerant taxa with visible *CAX1* expression, the *CAX1* gene could be cloned, sequenced, and examined for obvious defects (e.g. deletions, nonsense mutations) that would prevent normal CAX1 function. Third, *CAX1* genes cloned from serpentine-adapted plants (and their nonserpentine sister taxa) could be directly tested for function in transgenic *A. thaliana* or yeast. Finally, quantitative trait locus mapping in crosses between serpentine-adapted and nonadapted plants could provide a comprehensive understanding of the genetic architecture of serpentine soil tolerance, with *CAX1* and the NSCC as candidate genes potentially capable of contributing to the serpentine-tolerant phenotype.

The genetic basis of additional components of the 'serpentine syndrome' (Jenny, 1980), such as drought tolerance, tolerance of low nitrogen and phosphorus availability, or tolerance to heavy metals in the soil, remains to be discovered. A complete picture of serpentine adaptation will require determining the relative contribution of each of these genetic factors to the evolution of serpentine tolerance in many plant taxa.

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