RESEARCH ARTICLE

The novel Arabidopsis thaliana svt2 suppressor of the ascorbic acid-deficient mutant vtc1-1 exhibits phenotypic and genotypic instability [v1; ref status: indexed, http://f1000r.es/o2]

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Abstract

Ascorbic acid is a potent antioxidant that detoxifies reactive oxygen species when plants are exposed to unfavorable environmental conditions. In addition to its antioxidant properties, ascorbic acid and its biosynthetic precursors fulfill a variety of other physiological and molecular functions. A mutation in the ascorbic acid biosynthesis gene VTC1, which encodes GDP-mannose pyrophosphorylase, results in conditional root growth inhibition in the presence of ammonium. To isolate suppressors of vtc1-1, which is in the Arabidopsis Columbia-0 background, seeds of the mutant were subjected to ethyl methanesulfonate mutagenesis. A suppressor mutant of svt2, with vtc1-1 wild-type levels of ascorbic acid and root growth similar to the wild type in the presence of ammonium was isolated. Interestingly, svt2 Arabidopsis Landsberg erecta features, although svt2 is delayed in flowering and has an enlarged morphology. Moreover, the svt2 genotype shares similarities with Ler polymorphism markers and sequences, despite the fact that the mutant derived from mutagenesis of Col-0 vtc1-1 seed. We provide evidence that svt2 is not an artifact of the experiment, a contamination of Ler seed, or a result of outcrossing of the svt2 mutant with Ler pollen. Instead, our results show that svt2 exhibits transgenerational genotypic and phenotypic instability, which is manifested in a fraction of svt2 progeny, producing revertants that have Col-like phenotypic and genotypic characteristics. Some of those Col-like revertants then revert back to svt2-like plants in the subsequent generation. Our findings have important implications for undiscovered phenomena in transmitting genetic information in addition to the Mendelian laws of inheritance. Our results suggest that stress can trigger a genome restoration mechanism that could be advantageous for plants to survive environmental changes for which the ancestral genes were better adapted.


**Associated Research Article**

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Introduction

L-Ascorbic acid (AA, vitamin C) is an important antioxidant with multiple functions in many species. It serves as a scavenger of reactive oxygen species generated under adverse environmental conditions. However, AA also influences flowering time and senescence, pathogen disease resistance, the biosynthesis of various plant hormones, and root development. This suggests that AA and some of its intermediates have functions in addition to its antioxidant properties.

Ascorbic acid biosynthesis in plants occurs predominantly through the d-mannose-3-phosphate pathway. Given the multifaceted functions of AA in plants, there is a need to advance our understanding of how plants regulate the biosynthesis and accumulation of AA. Arabidopsis thaliana mutants deficient in AA have provided important insights into the breadth of molecular and physiological functions of AA. One of the Arabidopsis mutants, vtc1-1, contains a defect in the AA biosynthetic enzyme GDP-mannose pyrophosphorylase. The mutant was originally generated by ethyl methanesulfonate (EMS) mutagenesis of Col-0 wild-type seed. The vtc1-1 mutant contains a point mutation in amino acid 22 that converts a conserved proline into a serine. The VTC1 gene has recently been shown to be a determinant of ammonium sensitivity in plants. In the presence of ammonium, vtc1-1 mutants exhibit strongly reduced root growth in comparison to the wild type, a phenomenon that is independent of AA deficiency. To better understand the mechanism through which VTC1 mediates conditional ammonium sensitivity, it is important to identify regulatory partners of VTC1. To accomplish this, we undertook a suppressor mutagenesis approach of vtc1-1 homozygous mutant seed in the hope of identifying vtc1-1 suppressor mutants that could then be isolated and studied.

One of the suppressor mutants isolated in the M1 generation, svt2 (suppressor of vtc1-1), contained wild-type AA levels and developed roots similar to the wild type in the presence of ammonium. However, while characterizing the mutant genotypically, we observed that it lost the original vtc1-1 mutation (i.e., svt2 contained the homozygous wild-type allele). Furthermore, we determined that svt2, although generated through EMS mutagenesis of Col-0 vtc1-1 mutant seed, was phenotypically and genotypically similar to Ler. Intriguingly, a small percentage of svt2 M1 plants produced offspring that have phenotypic and genotypic similarities to Col in the M2 generation. Even more remarkably, a small percentage of Col-like revertants in the M1 generation produced progeny that exhibited phenotypic and genotypic svt2 characteristics again in the M2 generation.

Phenotypic instability of Arabidopsis alleles affecting a disease resistance gene cluster has recently been reported. In their work, Yi and Richards described that exposure to EMS or through the generation of different F1 hybrids induced phenotypic instability in the bal and cpr1 mutant alleles. The authors later proposed that the high phenotypic instability is caused by a genetic mechanism.

The presented study focuses on describing and characterizing the Arabidopsis svt2 suppressor mutant and its phenotypic and genotypic behavior. After illustrating the phenotypic features of svt2, we investigate transgenerational changes in the phenotype and genome of svt2 and provide evidence that svt2 is a true mutant and not the result of an experimental artifact or contamination. Finally, we discuss our experimental findings in respect to the vtc1-1 mutant background and other reports that previously described similar phenomena of genome instability and restoration, and we briefly speculate on possible mechanisms of phenome and genome instability in svt2.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Col-0 wild type and the previously described vtc1-1 mutant (in the Col-0 background) were kindly provided by Patricia Conklin (SUNY Cortland, NY, USA). Ler-0 wild-type seed were obtained from The Arabidopsis Biological Resource Center (www.arabidopsis.org). Plants were grown in MetroMix 360 potting soil at 23°C at both day and night with a 16-hour photoperiod at 160 μmol photons m−2 s−1 (fluorescent bulbs).

For assessment of root growth, seed of the wild types and mutant lines were surface-sterilized (see below) and grown on basal full strength 1× Murashige and Skoog (MS) medium without vitamins (Cat.# MSP01, Caisson Laboratories, Inc., North Logan, UT), containing 1% PhytoBlend (Cat.# PTP01, Caisson Laboratories) in omni trays (Fisher Scientific, Pittsburgh, PA) as described. Sucrose was omitted from the tissue culture medium. The pH of the medium was adjusted with KOH to 5.7. Trays were sealed with two layers of 3M micropore tape (Fisher Scientific), put in vertical orientation, and placed in the growth chamber under long days (16 h light, 8 h dark) at 23°C and grown for 4 days at 4°C.

To assess AA content in leaf tissue, seeds of wild type and mutants were randomly sown on MetroMix 360 soil (BFG supplies Co., Burton, OH) in the same flat under the growth conditions described above. When plants were three weeks old, whole rosettes were harvested for the AA assay.

Seed-surface sterilization

Seeds were soaked for 1 min in 50% ethanol, followed by washing the seeds in 50% bleach plus 0.01% sodium dodecyl sulphate for 6 min. Finally, seeds were rinsed six times with sterile water and stored in 0.1% sterile Phytoblend agar for 2 d at 4°C.

Ethyl methanesulfonate mutagenesis

Seeds of homozygous vtc1-1 Arabidopsis thaliana (Col-0 background) were mutagenized with 0.2% ethyl methanesulfonate as described. Approximately, 1200 M0 seed were stratified for 4 days at 4°C in 0.1% agar, sown on MetroMix soil and grown as above. Plants were screened for wild-type AA levels using the nitroblue tetrazolium assay. Additional suppressor mutants were isolated by pooling seeds generated from M1 plants. Putative mutants were isolated and allowed to self-pollinate to obtain seed.

Pollen grain analysis and microscopy

Pollen was taken from 4.5-week-old flowering plants of Col-0 and Ler wild type and vtc1-1 and svt2 M1 mutants, mounted in glycerol, and photographed using bright field settings on a Nikon E800 microscope equipped with a CoolSNAP cf CCD camera (Photometrics, Tuscon, AZ, USA).
Figure 1. Isolation of svt2. To isolate vtc1-1 suppressor mutants, homozygous vtc1-1 seed (in the Col-0 genetic background) were exposed to chemical mutagenesis using ethyl methanesulfonate (EMS). Over 1000 mutagenized seed (M0) were planted on soil and screened for wild-type levels of ascorbic acid content. The only mutant isolated in the M0 generation containing recovered ascorbic acid levels was svt2. The mutant was allowed to self-fertilize and was characterized phenotypically and genotypically in subsequent generations.

Genomic DNA isolation
Genomic DNA was isolated from rosette leaves following a previously described protocol. In case of genomic DNA isolation from vtc1-1 seeds, a small amount of dried seeds was crushed and the extraction procedure described previously was followed. Primers for the VTC1 gene and for the Insertion/Deletion (InDel) polymorphisms were designed using sequence data available on The Arabidopsis Information Resource (TAIR) database (www.arabidopsis.org). Polymerase chain reaction (PCR) was used to amplify fragments of the VTC1 gene for sequencing and to assess InDel polymorphisms. Sequences of primers used for sequencing and InDel analysis are summarized in Table 1. PCR reactions were run on 1.0 % agarose gels stained with ethidium bromide.

Gene copy analysis using qPCR
Quantitative PCR reactions were set up to measure gene copy number using 2.5 pmole gene-specific primers, 300 ng of genomic DNA diluted in DNase/RNase free water, and iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) for a total volume of 10 μL. Reactions without template were used as negative controls. Each single copy reaction was set up in triplicate and run in a Bio-Rad iCycler for 40 cycles. Threshold cycles (Ct) were calculated using iQ software (Bio-Rad).

Primer efficiencies (E) were calculated using cDNAs synthesized from RNA isolated from Col-0 plants as previously described. cDNA samples were serially diluted across three orders of magnitude. Serial dilutions were amplified in triplicate using the same protocol as for the copy number experiment. The Ct's of each triplicate were averaged and plotted against the dilution factor. A linear trend was fitted to the data and the slope of this trend was used to calculate E for each primer with the formula: E=10^{(1/-slope)}.

Copy number of VTC1 (AT2G39770) was calculated using the formula: Reported Quantity (RQ) = 1/E^T normalized to the RQ of a known single copy gene (PAD4, AT3G52430), VTC1 RQ was calculated from the average VTC1 RQ of three biological replicates per genotype and was normalized to the average RQ of PAD4 from three replicates of each respective genotype, all run in the same reaction plate.

Sequence analysis
PCR products were purified using the Qiagen Miniprep Kit. Dye-terminator based DNA sequencing was performed at the Genomics Facility in the Department of Biology at West Virginia University. Sequence alignments were performed using the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Ascorbic acid quantification
To screen mutants, AA levels were analyzed qualitatively in small pieces of two-week-old rosette leaves using the nitroblue tetrazolium assay previously described. The AA content was determined in whole rosettes of three-week-old plants using the iron reduction assay.

Statistical analysis
Experiments were performed at least three times. Figures represent individual experiments. Data were expressed as mean values ± SE. P values were determined by Student’s t test analysis.

Table 1. Forward (F) and reverse (R) sequences of primers used in analyzing the VTC1 gene and for amplying five Col/Ler Insertion/Deletion (InDel) polymorphisms.

| Primer Name | 5’ 3’ |
|-------------|------|
| VTC1G1F     | AAA AAT TCG TTC TAG ATG GAT GCT |
| VTC1G1R     | ATG GCT GTA A ATG AAG AGA T     |
| VTC1G2F     | GAA CCC TTG TCT CTA AAA TA      |
| VTC1G2R     | CAA ATC CCA TAA TCT GTT CC      |
| VTC1G3F     | GAA TTT TGC TTA CTT CTC TCT     |
| VTC1G3R     | TGG ATG CAA CCG ACA CAA AAC AAT |
| VTC1G4F     | ACA TTT TGA GCA GCT GAT GGT     |
| VTC1G4R     | AGG TAA GAA CTG GCA GAC TAA AG  |
| VTC1G5F     | TCG TCT GAG ACC ATT GAC T       |
| VTC1G5R     | GAG GCT TCC CCA CCG TGA GTT     |
| VTC1G6F     | CAA GCT GCA AAT CAA AAT CAC T   |
| VTC1G6R     | GCG CTG CTG CAA TCT CAG G       |
| VTC1G7F     | ACA AAT TTC AGC GTC GGG AAG C   |
| VTC1G7R     | TGG TTA ATT TGG CAG GAG A       |
| VTC1G8F     | CAA GGG CTC TAT GCT ATG GTG     |
| VTC1G8R     | GCG TTT TQA TTA ATG CTT ATT C   |
| VTC1G9F     | GCG TGT ATC TCG AGC AGT ATC AT  |
| VTC1G9R     | GTG GAG GGA AGT TAA GGG TAT T   |
| Indel1      | 1450919F | ATC GGT TTG TAA TCT CTG TCC A  |
| Indel1      | 1450919R | TAT GCG TCC CCA AAT TTG TTA TCT C |
| Indel2      | 451470F  | GGA GAC CCA AAC TGC TAT TAC A  |
| Indel2      | 451470R  | AAC CGC CTC CAT TTG CAC CTT ATC |
| Indel3      | 469762F  | GTC ACC GAG TTT TGG TTT GCT CAT |
| Indel3      | 469762R  | GCT CAC GAG TTT CTG TTT CGG TGG TAG |
| Indel4      | 449053F  | GAA AGA AAG CAG CGA AAC ACA     |
| Indel4      | 449053R  | GCC CAT GCC CAT ACA CTG A       |
| Indel5      | 455100F  | ACT TGC TTA ATC GTT TCT TTG TA  |
| Indel5      | 455100R  | GCC CAC TCG TAT TCG TAG TTG    |
Results

Isolation of svt2

Our laboratory is interested in understanding how the \textit{VTC1} gene, which is essential for the biosynthesis of GDP-mannose and AA, is regulated. This would help deciphering the pleiotropic phenotypes displayed by \textit{vtc1-1}, including its hypersensitivity to ammonium\textsuperscript{8–11}. We employed a gene suppressor analysis with the goal of identifying novel genes that interact or regulate \textit{VTC1}. Seed of the \textit{vtc1-1} mutant, which is in the Col-0 genetic background\textsuperscript{14}, were subjected to chemical mutagenesis using EMS\textsuperscript{18}. One thousand and one hundred mutagenized \textit{vtc1-1} seeds (\textit{M\textsubscript{0}} generation) were planted onto soil and screened for recovered (wild-type) leaf AA content using the qualitative nitroblue tetrazolium test\textsuperscript{19}. One of the mutants exhibited wild-type AA levels in the \textit{M\textsubscript{0}} generation. This mutant was named \textit{svt2} (\textit{suppressor of vtc1-1} 2), isolated, and further characterized. The mutant was allowed to self-fertilize and seeds from the plant were collected (\textit{M\textsubscript{1}} generation) (Figure 1).

Note that we isolated additional suppressor mutants by pooling \textit{M\textsubscript{2}} seed and by screening for long roots on 1× Murashige and Skoog (MS) medium containing ammonium. Six suppressor mutants were identified among 2000 plants. \textit{M\textsubscript{3}} seed were collected and screened for long roots again to test for segregation. \textit{M\textsubscript{4}} progeny of one line had all long roots, whereas the other five lines segregated in a ratio of three plants producing long roots, and one plant having short roots. Figure 2 summarizes data of four of these suppressor mutants, with D3–4 homogenously producing long roots, whereas D3–3, D3–7, and D3–15 developed long and short roots in a 3:1 ratio. As is illustrated in Figure 2A, these suppressor mutants developed roots that were significantly longer than those of the Col-0 wild type. Analysis of the total AA content revealed that the suppressor D3–4 had an AA content comparable to the Col-0 wild type, whereas that of \textit{vtc1-1} was only approximately 40% of that of the wild type\textsuperscript{14,15}.

Finally, sequence analysis of these four suppressor mutants demonstrated a lack of the \textit{vtc1-1} mutation (Figure 2C). Except for the assessments described above, these suppressor mutants were not yet characterized further.

\textit{svt2} has similarities with the \textit{Ler} phenotype, but has also phenotypic characteristics that are distinct from \textit{Ler}

The first observation we made when characterizing \textit{svt2} \textit{M\textsubscript{1}} plants was that \textit{svt2} exhibited a phenotype reminiscent of the \textit{Ler} ecotype with the characteristic round leaves and erect morphology when compared to Col (Figure 3A). However, \textit{svt2} also had features that were distinct from the \textit{Ler} phenotype, including overall enlarged vegetative and reproductive morphology (insets of rosettes and flowers in Figure 3A). In addition, \textit{svt2} was strongly delayed in flowering compared to the Col-0 and \textit{Ler}-0 wild types and the \textit{vtc1-1} mutant (Figure 3A, 3B). Primary inflorescences in four-week-old plants were 1.4-times significantly longer in the \textit{vtc1-1} mutant and approximately twice as long in the \textit{svt2} mutant.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Phenotypic and genotypic characterization of additional \textit{vtc1-1} suppressor mutants. (A) Root length in seven-day-old seedlings grown on 1× MS. Bars represent means ± SE of 18–73 individuals. Since D3-4 homogenously produced long roots, all individuals were included in the calculations. As D3-3, D3-7, and D3-15 developed long and short roots in an approximate 3:1 ratio, only individual seedlings that produced long roots were included in the calculations. (B) Total ascorbic acid content per gram fresh weight in whole rosettes of three-week-old plants. Bars represent means ± SE of three (Col-0 and \textit{vtc1-1}) or 24 individual replicates. *** \textit{P} < 0.001 by Student’s \textit{t}-test indicates significant differences in comparison to the Col-0 wild type. (C) Sequences of the Col-0 wild type, the \textit{vtc1-1} mutant and four suppressor mutants. The arrow points to the \textit{vtc1-1} mutation, a conversion of cytosine to a thymine.}
\end{figure}
long in the Ler-0 wild type compared to the Col-0 wild type. In svt2 mutant plants, however, buds of primary inflorescences only began to emerge when plants were four weeks old (Figure 3A, 3B). The flowering data are consistent with previous reports, with Ler-0 wild type entering the reproductive phase before Col-0 wild type. An early flowering phenotype of vtc1-1 has been reported previously

The AA content in svt2 was similar to levels quantified in Col-0 and Ler-0 wild types, whereas vtc1-1 contained approximately 30% of the AA content as expected

We therefore assessed five additional Insertion/Deletion (InDel) polymorphisms randomly chosen across the five Arabidopsis chromosomes (Table 1) in svt2 compared to the Col-0 and Ler-0 wild types and sequenced the entire VTC1 gene and the promoter region tested. Our data show that the PCR products generated for those five InDel using svt2 genomic DNA had the same electrophoretic mobility as those produced from Ler-0 genomic DNA (Figure 7)

Moreover, sequence analysis of the VTC1 gene and promoter region revealed that svt2 contained a 283 bp insertion in the VTC1 promoter (Figure 5C). The insertion is highlighted in gray in Figure S1. Note additional single nucleotide polymorphisms as indicated by upright arrows in Figure 5C and Figure S1. When we aligned the VTC1 gene sequence obtained from svt2 with that of the vtc1-1 mutant, the VTC1 Col-0 gene sequence deposited in the TAIR database, and the VTC1 Ler GenBank sequence, the VTC1 gene sequence in svt2 shared similarities with Ler (upright arrows in Figure 5C, Figure S1) and Col (arrows pointing down in Figure S1). However, note that there are sequences that are unique to svt2 and are not shared between Col, vtc1-1 or Ler (arrowheads in Figure S1). Finally, note the overlap in sequences between Col, vtc1-1, svt2 and Ler on the 5’ end of the sequence flanking the insertion (at approximately base pair 190); see left-facing horizontal black arrow in Figure S1 compared to the sequence flanking the 3’ end of the DNA sequence insertion (starting at base pair 2273); see right-facing horizontal black arrow in Figure S1.

Finally, most intragenic suppressor mutants still contain the original mutation in addition to the suppressor mutation. Therefore, we expected that the vtc1-1 mutation is still present in svt2. However, our sequencing analysis demonstrated that svt2 did not contain the vtc1-1 mutation anymore and that the mutation reverted back to the homozygous wild-type allele (Figure 5D; green shading in Figure S1).

In summary, our data demonstrate that svt2 shares DNA sequence similarity with Col and Ler, but also contains DNA sequences that are unique to this mutant. This is particularly remarkable because svt2 was generated in the vtc1-1 Col-0 background. Also, svt2 did not contain the original vtc1-1 mutation anymore. Although our data already argue against svt2 being a result of an artifact of the experiment or a contamination with Ler, we analyzed subsequent svt2 generations and discovered additional characteristics that are unique to svt2.

svt2 exhibits phenotypic and genotypic instability

Our initial observations revealed that approximately 10% of svt2 M1 plants displayed a Col-like phenotype. Therefore, we planted svt2 M1, M2, and M3 progeny to check whether this result could be repeated and to determine segregation ratios (Table 3). Additionally,
Figure 3. Phenotypic characterization of svt2. (A) Flowering phenotype of four-week-old Col-0 wild type, the vtc1-1 and svt2 mutants and the Ler-0 wild type. Insets show rosette phenotypes of the four genotypes when plants were three weeks old and the flower phenotype of six-week-old plants, respectively. (B) Primary inflorescence length when plants were four weeks old. Bars represent means ± SE of eight individual replicates. (C) Total ascorbic acid content per gram fresh weight in whole rosettes of three-week-old plants. Bars represent means ± SE of three individual replicates. (D) Root length in seven-day-old seedlings grown on 1× MS. Bars represent means ± SE of 30-90 individuals. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test indicate significant differences in comparison to Col-0 and Ler-0 wild type, respectively.
we investigated whether \textit{svt2} progeny that were phenotypically Col-like revertants would produce \textit{svt2} (Ler-like) offspring in the next generation.

As summarized in Table 3, revertants could only be detected when a relatively large population was planted. In the \textit{svt2} \textit{M} \textit{1} generation, only 1\% of Col-like revertants were detected. In contrast, 8–10\% of \textit{svt2} \textit{M} \textit{2} plants displayed a Col-like phenotype, whereas no revertants were detected in the \textit{svt2} \textit{M} \textit{3} generation. These Col-like revertants were isolated and seeds were collected from individual plants and the phenotype of the progeny in the \textit{M} \textit{2} generation was assessed in some examples. In most cases, reversion appeared to be stable, i.e., once \textit{svt2} plants reverted, displaying a Col-like phenotype in the \textit{M} \textit{2} generation, their \textit{M} \textit{3} progeny continued to appear as Col-like plants. This was the case for the \textit{M} \textit{2} progeny of the A8 and G7 plants listed in Table 3. However, out of 63 progeny from the K1 revertant plant, one reverted back to a \textit{svt2}-like phenotype (Table 3), i.e., the K1 double revertant switched from \textit{svt2} phenotype in the \textit{M} \textit{2} generation to a Col-like phenotype in the \textit{M} \textit{3} generation, and then reverted back to a \textit{svt2}-like phenotype in the \textit{M} \textit{4} generation. Note that only a small number of progeny was planted. In a second experiment, the \textit{svt2} Col R1 revertant produced 20 individuals displaying a \textit{svt2}-like phenotype (Table 3). This represents a larger reversion percentage than in the K1 double revertant (22.7\% vs. 1.6\%). This may be explained by the genotypic make-up of the Col-like reverted parents and will be presented in the next section. Figure 8 illustrates the phenotypic appearance of three examples of \textit{svt2} → Col single revertants (Col R1, Col R2, K1 Col R) and a \textit{svt2} → Col → \textit{svt2} double revertant (K1 Col R \textit{svt2} R).

Next we tested whether a Col-like revertant phenotype correlated with a Col-like genotype. Likewise, we would expect that a \textit{svt2} → Col → \textit{svt2} double revertant phenotype corresponds with \textit{svt2}-like genomic markers. To check this we isolated genomic DNA from Col-0 and Ler-0 wild types, \textit{svt2}, \textit{vtc1-1} and revertant mutants, and PCR-amplified the five randomly selected InDel polymorphisms plus the InDel polymorphism in the \textit{VTC1} promoter (Table 1). In all cases but the \textit{svt2} \textit{M} \textit{1} Col R1 revertant, the \textit{svt2}-like revertant plants (labeled \textit{svt2} \textit{M} \textit{1} Col revertants 1 through 5) produced PCR products that were of the same electrophoretic mobility as the PCR products generated using Col-0 wild-type genomic DNA. In contrast, \textit{svt2} \textit{M} \textit{2} plants and \textit{svt2} \textit{M} \textit{3} plants that displayed an \textit{svt2} phenotype, gave rise to PCR products that were of the same electrophoretic mobility as those of the \textit{Ler} wild type (Table 4, Figure 9). In addition, the double revertant plant K1 (labeled \textit{svt2} \textit{M} \textit{1}, K1 Col R) was genotyped in both its \textit{M} \textit{2} and \textit{M} \textit{3} generations. The K1 plant produced InDel PCR products similar to those of the Col-0 wild type in the \textit{M} \textit{2} generation. However, the \textit{M} \textit{3} generation that displayed \textit{svt2}-like morphology produced PCR products that were comparable to the \textit{InDel} PCR products generated using \textit{Ler} genomic DNA (Table 4). The \textit{svt2} \textit{M} \textit{1} Col R1 (highlighted in red in Table 4) is intriguing, because it appears to contain DNA that is similar to both Col and \textit{Ler} genomic DNA. This suggests the presence of chimeric genome sectors, which may explain the higher percentage of Col-like revertants compared to \textit{svt2} \textit{M} \textit{1} K1.
Figure 5. Genotypic characterization of svt2. (A) VTC1 Col-0 gene model. Light green box indicates VTC1 gene promoter region, light blue rectangles indicate 5’ and 3’ UTRs, dark blue rectangles indicate exons, and lines indicate introns. Shown is the location of the vtc1-1 mutation within the first exon, primer locations, and polymorphism insertion of 283 bp in Ler-0 VTC1. (B) PCR amplification of the VTC1 promoter region in the Col-0 wild type, vtc1-1 and svt2 mutants and Ler-0 wild type. (-) indicates negative control, no DNA. (C) Partial sequence alignment of the VTC1 promoter region from the TAIR database (Col-0), sequenced Col-0 wild type, vtc1-1 and svt2 mutants, sequenced Ler-0 wild type and the Ler-0 sequence obtained from GenBank. The alignment shows the sequence insertion in the svt2 mutant, the Ler-0 wild type and the GenBank sequence. Arrows indicate single nucleotide polymorphisms between the Ler-0 and Col-0 sequence. (D) Point mutation in vtc1-1, a conversion from a cytosine to a thymine.

Figure 6. Molecular characterization of svt2. Amplification of the VTC1 gene including ~500 bp of the promoter region using a series of nine, overlapping primers (G1F+R through G9F+R) in both Col-0 wild type and svt2 M1 mutant genomic DNA. The last lane in each gel contained a negative control (water instead of DNA). Red arrows indicate the different sized PCR products using the same primer set.
Col R. Note that the PCR results are in line with the sequencing analysis of the revertants. That is, Col-like revertants and svt2-like revertants share sequence similarity with Col-0 and Ler wild type, respectively (Figure S2).

Taken together, these data suggest (i) transgenerational phenotypic and genotypic instability in svt2, and that (ii) svt2 offspring do not segregate in a Mendelian fashion. In an attempt to obtain first insights toward a mechanism that is causing this genotypic instability, we investigated whether transgenerational epigenetic inheritance could play a role.

Genome instability in svt2 does not appear to be triggered by a transgenerational epigenetic mechanism

To investigate whether genome instability is caused by transgenerational epigenetic inheritance in the svt2 mutant, we performed reciprocal crosses between svt2 mutants and Col-0 wild-type plants. It is possible that through the EMS mutagenesis of vtc1-1 seeds, genes involved in the regulation of epigenetic alterations were altered, whereby their activity was affected. There is increasing evidence in both plants and animals that epigenetic marks are not always cleared between generations. Incomplete erasure at genes associated with a measurable phenotype results in unusual patterns of inheritance

### Table 3. Summary of revertant data

The table summarizes the number of plants screened in each of three svt2 generations (M₁, M₂, and M₃), screens of revertant progeny from Col-like revertants (A8, G7, K1), and the revertant progeny of a Ler-like line (K1 Col R svt2 R). The percent reversion is shown in the last column. Although the number of progeny plants tested is relatively large, some lines did not give rise to revertant progeny. R denotes revertant. *indicates mutant plants that were also analyzed genotypically (see Table 4).

| Experiment | Generation | Total # of plants | # of phenotypic revertants | % reversion |
|------------|------------|--------------------|---------------------------|-------------|
| 1 svt2 M₁ | 63         | 0                  | 0                         | 0           |
| svt2 A8 Col R M₁ | 78        | 7 (Col phenotype) | 8.97                      |
| svt2 G7 Col R M₁ | 64        | 0                  | 0                         |
| svt2 K1 Col R M₁ | 64        | 1 (svt2 phenotype) | 1.58                      |
| svt2 K1 Col R svt2 R M₁ | 63 | 0                  | 0                         |
| svt2 M₂ | 96         | 0                  | 0                         | 1.04        |
| svt2 M₂ | 96         | 1 (Col phenotype) | 1.04                      |
| svt2 Col R1 M₂ | 88        | 5 (Col phenotype) | 8.06                      |
| svt2 Col R4 M₂ | 96        | 20 (svt2 phenotype) | 22.73                     |
| svt2 M₃ | 96         | 10 (Col phenotype) | 10.42                     |
from one generation to the next, termed transgenerational epigenetic inheritance\textsuperscript{22,23}. Therefore, analysis of the progeny of the reciprocal crosses is expected to provide some first insights on the possibility of transgenerational epigenetic inheritance that is transmitted maternally. If this were the case, only progeny of crosses with a maternal \textit{svt2} donor should have a \textit{svt2}-like phenotype. To determine the genotypes of the F\textsubscript{1} progeny of the reciprocal crosses, we performed another InDel polymorphism assay as described above. In addition, progeny were also screened using the \textit{VTC1} InDel promoter polymorphism. Table 5 contains a summary of the InDel screen for progeny from each reciprocal cross. In all but six of the progeny from the reciprocal crosses, PCR products similar to those obtained using \textit{Col} and \textit{Ler} genomic DNA, respectively, were generated, suggesting that the F\textsubscript{1} of the reciprocal crosses were heterozygous. A similar result was obtained for the \textit{VTC1} promoter polymorphism marker in all reciprocal crosses. Note, however, that for some polymorphisms and irrespective of whether \textit{svt2} or \textit{Col}-0 served as female or male donor, respectively, PCR products comparable to those obtained using \textit{Ler}-0 wild-type DNA were prevalent (highlighted in red in Table 5). This is surprising because heterozygosity was expected at all loci. This suggests that some parts of the genome were not inherited equally from both parents. Taken together, these results suggest that maternal epigenetic inheritance may not be the cause of genome instability in \textit{svt2}. However, at some loci \textit{svt2}-like alleles dominate over \textit{Col}-0.

### Discussion

The \textit{svt2} mutant was initially identified as a putative suppressor of the AA-deficient \textit{Arabidopsis} mutant \textit{vtc1-1}, as was evident in wild-type levels of AA (Figure 3C) and recovered root development in the presence of ammonium (Figure 3D). However, \textit{svt2} manifests other interesting characteristics, including genotypic and phenotypic instability. These unique features could aid in our understanding of the complex mechanisms controlling genome instability and restoration.

\textit{svt2} is a novel \textit{Arabidopsis} mutant and not a result of an experimental artifact, seed contamination, or outcrossing

Several lines of evidence support our findings that \textit{svt2} is a novel mutant. First, \textit{svt2} was the only suppressor mutant isolated among over 1000 EMS-mutagenized \textit{M\textsubscript{2}} seeds to show unique phenotypic characteristics. Astonishingly, our genetic analysis revealed that both maternal and paternal alleles were affected in five randomly selected InDel polymorphism loci, the newly discovered InDel polymorphism in the \textit{VTC1} promoter, and additional SNPs (Figure 5B–D, Figure 6, Figure S1). These data demonstrate that \textit{svt2} has acquired new characteristics, presumably as a result of EMS mutagenesis, and that \textit{svt2} is neither \textit{Col} nor \textit{Ler}. These data also argue against \textit{svt2} being an experimental or PCR artifact.

Second, a number of data provide strong arguments against seed contamination. (1) With high reproducibility, descendents of the original \textit{svt2} mutant produce offspring revertants with Col-like features (Table 3, Table 4; Figure 8, Figure 9). (2) One of the Col-like revertants, \textit{svt2} \textit{Col R M\textsubscript{1}}, exhibited heterozygosity at some of the InDels tested (Table 4). (3) One of those Col-like revertants, \textit{svt2} \textit{K1 Col R M\textsubscript{1}}, produced progeny that reverted

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**Figure 8. Phenotype of \textit{svt2} revertants.** Plants were three weeks old when photographs were taken. Top row represents controls, \textit{Col-0} wild type, \textit{vtc1-1} and \textit{svt2} mutants, and \textit{Ler-0} wild type. Bottom row represents three Col-like revertants, \textit{svt2} \textit{Col R M\textsubscript{1}}, \textit{svt2} \textit{Col R2 M\textsubscript{1}}, \textit{svt2} \textit{K1 Col R M\textsubscript{1}}, and a double revertant, \textit{svt2} \textit{K1 Col R svt2 R M\textsubscript{1}}. R stands for revertant.

**Table 4. Summary of PCR-based molecular genotypes.** With the exception of \textit{svt2} \textit{Col R1 M\textsubscript{1}}, where \textit{Col} and \textit{Ler} markers and one heterozygous marker were found (highlighted in red), phenotype matched genotype. That is, a Col-like phenotype correlated with the presence of \textit{Col} polymorphisms, while a Ler-like phenotype correlated with \textit{Ler} polymorphisms. \textit{C}, \textit{L}, and \textit{H} refer to \textit{Col}, \textit{Ler}, or heterozygous, respectively. R denotes revertant. n.d., not detected.

| Genotype        | InDel 1   | InDel 2   | InDel 3   | InDel 4   | InDel 5   | G1F + G2R VTC1 |
|------------------|-----------|-----------|-----------|-----------|-----------|----------------|
| \textit{Col-0} WT| C C C C C C |
| \textit{vtc1-1}  | C C C C C C |
| \textit{Ler-0} WT| L L L L L L |
| \textit{svt2} M\textsubscript{1} | L L L L L L |
| \textit{svt2} M\textsubscript{2} | L L L L L L |
| \textit{svt2} \textit{Col R1 M\textsubscript{1}} | C L C H C C |
| \textit{svt2} \textit{Col R2 M\textsubscript{1}} | C C C C C C |
| \textit{svt2} \textit{Col R3 M\textsubscript{1}} | C C C C n.d. C |
| \textit{svt2} \textit{Col R4 M\textsubscript{1}} | C C C C C C |
| \textit{svt2} \textit{Col R5 M\textsubscript{1}} | C C C C C C |
| \textit{svt2} \textit{K1 Col R M\textsubscript{1}} | C C C C C C |
| \textit{svt2} \textit{K1 Col R svt2 R M\textsubscript{1}} | L L L L L L |

Summary of PCR-based molecular genotypes

1 Data File
http://dx.doi.org/10.6084/m9.figshare.103772

Summary of PCR-based molecular genotypes

1 Data File
http://dx.doi.org/10.6084/m9.figshare.103773
back to svt2-like plants (Table 3, Table 4; Figure 8, Figure 9). (4) We were unable to obtain true F₁ heterozygotes in all svt2/Col-0 reciprocal crosses (Table 5). (5) Delayed flowering and enlarged morphology phenotypes argue against the fact that svt2 is a result of a Ler-0 wild-type seed landing on the flat during the

**Figure 9.** Insertion/Deletion polymorphism analysis in svt2, Col-0, Ler-0, and revertants. PCR amplification of the Col/Ler VTC1 promoter polymorphism in svt2 plants and svt2 revertant (R) plants, amplified with the VTC1 G1F and G2R primers. (-) indicates negative control, no DNA.

**Table 5.** Reciprocal crosses between svt2 and Col-0 wild-type lines. Molecular analysis of the InDel polymorphism markers showed evidence of cryptic but persistent homozygosity, irrespective of the direction of the sexual cross (highlighted in red). However, heterozygosity was expected at all loci.

| Female x Male crosses | InDel 1 450919 | InDel 2 451470 | InDel 3 469762 | InDel 4 449053 | InDel 5 455100 | G1F + G2R VTC1 |
|-----------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| svt2 x Col-0 F₂ 1     | H              | H              | L              | H              | H              | H              |
| svt2 x Col-0 F₂ 2     | H              | H              | H              | H              | H              | H              |
| svt2 x Col-0 F₂ 3     | H              | H              | H              | H              | H              | H              |
| svt2 x Col-0 F₂ 4     | H              | H              | H              | H              | H              | H              |
| Col-0 x svt2 F₁ 1     | H              | H              | L              | H              | H              | H              |
| Col-0 x svt2 F₁ 2     | H              | H              | L              | H              | H              | H              |
| Col-0 x svt2 F₁ 3     | H              | H              | L              | H              | H              | H              |
| Col-0 x svt2 F₁ 4     | H              | H              | L              | H              | H              | H              |

Repeated PCR reactions of reciprocal crosses between svt2 and Col-0 wild-type lines

1 Data File
http://dx.doi.org/10.6084/m9.figshare.103775

Reciprocal crosses between svt2 and Col-0 wild-type lines

1 Data File
http://dx.doi.org/10.6084/m9.figshare.103776

Repeated reciprocal crosses between svt2 and Col-0 wild-type lines

1 Data File
http://dx.doi.org/10.6084/m9.figshare.103777

Reciprocal crosses between svt2 and Col-0 wild-type lines

1 Data File
http://dx.doi.org/10.6084/m9.figshare.103774
initial planting of the vtc1-1 M₄ mutagenized population. There is the possibility of a Ler seed contamination of the vtc1-1 seed stock used for EMS mutagenesis. Although we have sequenced the vtc1-1 seed stock used for this experiment and confirmed that it is homozygous for the vtc1-1 mutation, one could argue that sequencing the seed stock may not be a sensitive enough method to rule out contamination with a few Ler seed. We performed many other experiments using this very same seed stock and never observed Ler-like plants among the vtc1 population. However, arguments (1) through (4) above speak most compellingly against seed contamination.

Third, the following experimental evidence argues against the possibility that svt2 was generated by cross pollination of vtc1-1 mutant plants with Ler wild-type plants. (1) If svt2 were generated by Ler cross-pollination, the InDel polymorphism markers tested using svt2 genomic DNA should have indicated heterozygosity. This, however, was not the case (Table 4). (2) While svt2 shares phenotypic and genotypic characteristics with Ler and Col, it also has unique features (Figure 3A, Figure S1). (3) svt2 exhibits phenotypic and genotypic instability, causing the appearance of revertants with persistent reproducibility. (4) Ler plants were not grown in our growth chambers at the time of the mutagenesis experiment. Furthermore, svt2 was isolated by placing Aracons over the mutant plant to allow self-fertilization and seed production.

**Possible causes of genome instability in svt2**

Our results are indicative of genome instability in svt2. Genome instability may be a result of polyploidy. Polyploids can arise from genome duplication (autopolyploids) or interspecific hybridization (allopolyploids). Our data suggest that svt2 does not contain multiple sets of chromosomes, because VTC1 occurs as a single copy gene in svt2 and vtc1-1 mutants as well as the Col-0 and Ler-0 wild-type controls (Table 2). Furthermore, extra DNA must be replicated with each cell division. Therefore, enlarged cell size is often associated with polyploids. The chemical mutagenesis of vtc1-1 seed could have resulted in mutations, which may have led to increased ploidy levels in one, two, or all three meristem layers, L1, L2, and L3. However, only mutations in the L2 layer, which gives rise to the reproductive organs, are inherited. Polyploidy in the L2 layer is reflected in pollen size. While svt2 has an overall enlarged morphology (Figure 3A), its pollen size is comparable to that of the other three genotypes (Figure 4). This suggests that svt2 anthers are not polyploid. Finally, allopolyploids often display a greater degree of heterozygosity, low fertility, and low embryonic viability. This, however, is not the case in svt2. The fact that svt2 is fertile and that its enlarged morphology is inheritable from one generation to the next suggests that svt2 is neither a somatic nor a gametic polyploid. Thus, it is therefore unlikely that polyploidy in svt2 contributes to genome instability. This is supported by Ruffio-Chable and co-workers, who reported that between 5% and 21% of F₁ hybrids in Brassica oleracea showed aberrant leaf phenotypes, despite normal ploidy levels.

Instead, we hypothesize that genome instability of svt2 was further aggravated by exposing the already unstable genome of vtc1-1 mutants to EMS. It has recently been shown that plants impaired in certain aspects of protection against reactive oxygen species have a higher incidence of spontaneous double-strand breaks. The AA-deficient vtc1-1 mutant has a three-fold higher spontaneous homologous recombination frequency and has a higher incidence of double-strand breaks (see below). Similar results were reported for the Arabidopsis thaliana flavonoid-deficient mutants tt4 and tt5. One may speculate that through the high level of stress induced by EMS, a yet unknown mechanism of genome restoration was turned on. In fact, genome alterations in soybean and flax in response to environmental stress have been reported previously. In the process of soybean cell culture, massive specific changes in numerous genome-wide loci were observed. It was suggested that this genetic variation is a consequence of specific recombinational events. Similarly, in flax a single-copy 5.7 kilobase DNA fragment that was not present in the parent line appeared in genotrophs in response to particular growth conditions.

**Possible mechanisms of genome restoration in svt2**

The experimental evidence described in this work raises the question as to what mechanism is responsible for the loss or reintroduction of genomic DNA sequences in the original svt2 mutant and its revertant offspring. Several mechanisms may be considered: activity of transposable elements, random mutations, unequal crossing over, gene conversion, double-strand breaks and recombination, and activity of an RNA cache.

Transposons are DNA elements capable of moving around the genome; movement is often associated with chromosome breaks and formation of unstable mutations, which revert frequently but often give rise to new phenotypes. Movement of transposable elements often occurs during meiosis and mitosis and is accelerated by genome damage. These represent conditions that are present in svt2. However, transposons have a variety of molecular features that do not apply to svt2. Transposons exist as multiple copies in the genome. A blast search of the VTC1 promoter insertion in svt2 did not return any other hits, indicating that the DNA sequence is not present in its entirety anywhere else in the genome. Additionally, transposon termini represent inverted repeats. This, however, is not the case in svt2 (Figure S1). A short, direct repeat of genomic DNA often flanks the transposon, leaving a “footprint”. Our sequencing analysis of the VTC1 promoter region in svt2 did not reveal any footprints, suggesting that transposon activity is not responsible for the insertion or loss of novel sequences in svt2 (Figure S1).

Random mutations caused by EMS mutagenesis could have activated an unknown mechanism in vtc1-1 seeds, giving rise to the phenotype and genome instability in svt2. This may explain the novel SNPs we detected in svt2 that are distinct from the vtc1-1 mutant and Col-0 and Ler-0 wild types (Figure S1). The disappearance of the vtc1-1 mutation in svt2 (Figure 5D, Figure S1) may also be explained by the introduction of a random mutation. However, it is possible that exposure of vtc1-1 seeds to EMS could have reversed the original vtc1-1 mutation to the wild-type sequence, as vtc1-1 was initially isolated in an EMS screen. Interestingly, Conklin and co-workers previously isolated two vtc1 alleles, vtc1-1 and vtc1-2, containing the exact same single cytosine to thymine point mutation at amino acid position 64 relative to the start codon, despite the fact that vtc1-1 and vtc1-2 mutants were isolated independently from different EMS-mutagenized pools. The authors
suggested that a limited number of mutations are tolerable in the VTC1 enzyme GDP-D-mannose pyrophosphorylase without causing embryo lethality. This is supported by the fact that several independently isolated cyt mutant alleles containing different amino acid mutations in VTC1 are embryo lethal\(^{34}\). To date, only the vtc1-1\(^{15}\) and hsn1 mutations\(^8\) have been isolated and reportedly do not cause embryo lethality. This suggests some form of allelic constraint that has been reported in *Arabidopsis* previously\(^{35,36}\). Furthermore, in the EMS screen the svt2 mutant was isolated, several other vtc1-1 suppressor mutants with restored root development in the presence of ammonium were identified. Sequencing analysis revealed that in all of these mutants the vtc1-1 mutation was restored to the wild-type allele, while the suppressor mutants neither exhibited a svt2-like phenotype nor did they produce revertants in the subsequent generation (Kempinski *et al.*, unpublished data).

Exposure to EMS or \(\gamma\)-radiation has been reported to induce high frequency phenotypic instability in the *Arabidopsis* disease resistance genes CPR1 and BAL, which map to the RPP5 locus\(^{16}\). Yi and Richards reported destabilization of phenotypes in both the bal and cpr1 mutants in more than 10\% of EMS-treated plants in the M\(_1\) generation. They also identified exceptions to simple Mendelian inheritance in the M\(_1\) generation. Phenotypic instability was also observed in bal \(\times\) cpr1 F\(_1\) hybrids. The authors suggested that the high degree of phenotypic instability in bal and cpr1 mutants is due to the fact that the RPR5 locus can adopt different metastable genetic or epigenetic states, whose stability is highly susceptible to mutagenesis and pairing of different alleles. Yi and Richards later reported that the phenotypic instability of bal mutants is caused mainly by gene duplication and hypermutation of the SNC1 gene\(^17\).

As observed in the cpr1 and bal mutants, we hypothesize that EMS treatment has destabilized the genome of svt2 by interrupting one or more mechanisms involved in genomic inheritance. A combination of unequal crossing over, gene conversion, double-strand breaks, DNA recombination, and/or the presence of an RNA cache template may explain the loss and reappearance of DNA sequences in svt2. Genome-wide non-Mendelian inheritance of extra-genomic information in *Arabidopsis* was reported in the hothead (hth) *Arabidopsis* mutant\(^{17}\). Self-fertilization of homozygous mutant plants resulted in approximately 10\% hth revertants, which were hth/HTH heterozygous, suggesting that the HTH gene was altered in the progeny. However, the authors also detected rare homozygous revertants HTH/HTH embryos, which must have inherited one of their two wild-type HTH genes from the maternal parent and could not have been a result of outcrossing. Inheritable genome-wide high-frequency gene homozygosity in early generations in rice has also been reported\(^8\). Lolle *et al.* postulated that these genetic restoration events are the result of a template-directed process that utilizes an ancestral RNA-sequence cache\(^7\). This hypothesis is supported by observations reported by Xu and co-workers\(^8\). Therefore, our genetic and phenotypic svt2 data, in conjunction with the observed higher occurrence of double-strand breaks and spontaneous homologous recombination frequency in vtc1-1, are in support of the RNA cache theory. Additional studies are needed to provide experimental support for this hypothesis.

**Conclusions**

We have isolated a novel *Arabidopsis* mutant that is capable of restoring genetic information that was not present in the chromosomal genome of its parents. We suggest that this ancestral information is present in some cryptic form that is accessible under extreme stress conditions. Genome restoration could be advantageous to plants that encounter environmental changes for which ancestral genes were better adapted. However, the mechanisms responsible for triggering and executing genome restoration remain to be determined. Double strand breaks, DNA recombination, and/or the activity of an RNA cache may be contributing factors. In the future, svt2 may serve as a model to study non-Mendelian inheritance and could provide insight into the evolution and diversification of *Arabidopsis* ecotypes.

**Abbreviations**

AA, ascorbic acid; EMS, ethyl methanesulfonate; InDel, Insertion/Deletion; MS, Murashige and Skoog.

**Author contributions**

CB and CFK conceived the study and designed the experiments. CFK, SVC, CS and CB conducted the experiments and analyzed the data. CB and CFK prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

**Competing interests**

No relevant competing interests disclosed.

**Grant information**

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*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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We would like to thank Dr. Patricia Conklin for providing vtc1-1 mutant and Col-0 wild-type seed. We also wish to thank Dr. Karen Weiler for allowing us to use her microscope and Dr. Rosana Schafer for providing a plate reader.
**Figure S1.** Sequence alignment of the VTC1 gene sequence of the Col-0 TAIR database, the vtc1-1, svt2 mutants, and the Ler-0 GenBank database. Horizontal arrows denote 5’ respectively 3’ flanking regions of the sequence insertion, which is highlighted in grey, in the VTC1 promoter region (between base pairs 1990 and 2273). Upright arrows indicate sequences shared between svt2 and Ler. Arrows pointing down denote sequences shared between svt2 and Col. Arrowheads point to sequences unique to svt2. Highlighted in yellow are the start and stop codons, respectively. Highlighted in green is the vtc1-1 mutation.
| Gene | Sequence 1 | Sequence 2 | Comparison |
|------|------------|------------|------------|
| Col-0_VTC1_gDNA_TAIR | ACCACTACATTTTTTTTTCCTT | ACCACTACATTTTTTTTTCCTT | Match |
| Ler-0_VTC1_gDNA_Genbank | ACCACTACATTTTTTTTTCCTT | ACCACTACATTTTTTTTTCCTT | Match |
| svt2 K1 Col R M3_G1F | ACCACTACATTTTTTTTTCCTT | ACCACTACATTTTTTTTTCCTT | Match |
| svt2 K1 Col R svt2 R M4_G1F | ACCACTACATTTTTTTTTCCTT | ACCACTACATTTTTTTTTCCTT | Match |

...
Figure S2. Sequence alignment of the VTC1 promoter InDel polymorphism sequence of the Col-0 TAIR database, the Ler-0 Genbank database, the svt2 K1 Col R M3 G1F revertant (Col-like phenotype) and the svt2 K1 Col R svt2 R M4 G1F revertant (svt2-like phenotype). Horizontal arrows denote 5’ respectively 3’ flanking regions of the sequence insertion, which is highlighted in grey, in the VTC1 promoter region in Ler-0 and svt2 K1 Col R M4, which exhibits an svt2-like phenotype. The svt2 K1 Col R M3 mutant has a Col-like phenotype and share sequence similarities with the Col-0 sequence. R denotes revertant.
Referee Responses for Version 1

David Oppenheimer
Department of Botany, University of Florida, Gainesville, FL, USA

Not Approved: 31 January 2013

Referee Report: 31 January 2013
In this paper, the authors characterize a suppressor of the vtc1-1 mutation, which they named svt2. The authors characterize the phenotype of the vtc1-1 mutants that carry the suppressor mutation and show that the plants have characteristics reminiscent of the Ler accession. Molecular characterization of the suppressed plants show that the suppressor mutation is apparently a reversion of the original vtc1-1 mutation, and surprisingly, have additional genomic signatures of the Ler ecotype as well as additional mutations.

There are several problems with the experimental methods used in this manuscript.

First, according to Figure 1 of the manuscript, the authors screened the M0 generation (the mutagenized seed) for wt ascorbic acid content. This is a significant problem for the subsequent mutant analysis in this manuscript. When Arabidopsis seed are mutagenized, the individual cells of the meristem on the seed are mutagenized independently. When the seed germinate, the plants are genetic mosaics. In addition, only those mutations in the L2 layer that gives rise to the germ cells will pass on the mutations. Therefore, it is highly unlikely that a seedling with wt ascorbic acid levels would be isolated from the M0, because it would take the accumulation of many independent mutations, each of which would need to lead to suppression of vtc1.

It is possible that a large sector of an M0 seedling could contain a suppressor mutation that leads to wt ascorbic acid levels, but this sector would have to include cells in the L2 layer for the mutation to be passed on to the next generation. Also, an Ler-like sector should be obvious on a mostly Col-0 plant. Nonetheless, a sector that included the L2 would lead to segregation of the phenotype in the M1 generation, because the cells in the sector would be heterozygous for the suppressor mutation.

Second, the concentration of EMS commonly used for mutagenesis in Arabidopsis (0.2%) is known to cause multiple mutations per genome. When one isolates a mutant of interest from an EMS screen, one should back-cross it at least once to allow these other mutations to segregate away. Otherwise, one may observe unexpected results when analyzing the mutant of interest due to the effects of these other mutations.

Third, when analyzing the sequence of the vtc1 gene in the original mutant and in the suppressor, svt2, the authors compared the sequence to the Ler and Col-0 sequences reported in Genbank and TAIR. Instead, the authors should sequence the vtc1 gene from their original vtc1-1 stock and the Ler accession that is present in their lab. This is because it is known that nucleotide polymorphisms arise regularly in lab
stocks such that a comparison between a lab stock of Col-0 and the reference sequence can show many
differences. Because the authors are reporting unexpected sequencing results, they should show the
actual sequence traces (from both strands) for the individual base pair differences highlighted in Figure
S1. It would be appropriate to show these sequence traces in the supplemental data. Showing the
sequencing traces for the base pair differences would demonstrate that the sequence differences are not
due to ambiguous base calling or other sequencing errors.

Fourth, the authors refer to the suppressed plants and their revertants has having a Ler-like or Col-like
phenotypes. Because the phenotype of vtc1 is lower ascorbic acid levels, and the putative suppressor
has wt ascorbic acid levels, the ascorbic acid levels in the revertants should be measured to show that
they are revertants, instead of relying on the Ler or Col phenotype.

Fifth, because seed and pollen contamination can explain the results, the authors need to explicitly state
the degrees to which they tried to eliminate these possible sources of contamination. Were plants of more
than 1 genotype grown together? Were seeds of more than 1 genotype collected in the same room? Was
soil stored where plants were setting seed? etc.

The single, Ler-like seedling found in the M₀ population can be explained as an Ler seed that
contaminated the Col-0 vtc1-1 seed stock used for the mutagenesis. This can be tested by sowing
several thousand Col-0 vtc1-1 seed from that seed stock, and screening them for the presence of any Ler
contaminants.

The results in Table 4 are the same as one would expect from seed contamination: the svt2 plants (with
the Ler phenotype) have all Ler markers, and the revertants (with the Col phenotype) have all Col
markers. The svt2 Col R1 M₂ plant highlighted in red shows the expected results if the parent of that plant
was heterozygous for Col/Ler. Again, the authors should state what extraordinary measures they used to
eliminate seed and pollen contamination.

Once these comments are addressed, the other unexpected results can be examined in a new light.

I have read this submission. I believe that I have an appropriate level of expertise to state that I
do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Competing Interests: No competing interests were disclosed.

Igor Kovalchuk
University of Lethbridge, Lethbridge, AB, Canada

Approved: 31 January 2013

Referee Report: 31 January 2013
Very unusual story. I am still puzzled how this is all possible. I can assume that original vtc1 line had some
Ler-1 background (may be from backcrosses). In this case it is possible that the seeds you started with for
mutagenesis are highly heterogeneous and some have Ler genomes still present. Now, such a severe
case of rearrangements due to combination of EMS and vtc1 background is unbelievable. I wonder why
other plants with even greater instability, such as ddm1 or msh2, have never had anything like this
reported. Maybe they have not looked for it hard enough? It would make sense to get the vtc1 mutant into rdr2 or rdr6 background (or both) and see whether this RNA cache plays any role - I would expect much lower chance of getting those revertants, same with reverse transcription mutants.

I understand that the event is rare – a single plant was produced – but it would really make the entire story stronger if several different plants were produced.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.

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**Andy Pereira**

Departments of Crop, Soil and Environmental Sciences, and Plant Pathology, University of Arkansas, Fayetteville, AR, USA

**Approved: 25 January 2013**

**Referee Report:** 25 January 2013

Very interesting experimental evidence of an inheritance phenomenon that is non-Mendelian and supports an RNA cache hypothesis. The data support the conclusions drawn, but some alternative explanations are put forth that can be addressed.

The EMS mutagenesis treatment of the vtc1-1 genotype yields a single suppressor svt2 mutant that turns out to be a revertant of the vtc1-1 mutation, and is homozygous. Since this screen was from ~1200 seed, it would be interesting to know if such revertant suppressor mutations are also be present in the original batch of vtc1-1 seed used for mutagenesis. Of course since the screen entails a tedious test of TTB on leaves of individual plants for AA content, it is not a recommended control test that should be done, but mechanistically the question remains if the locus is mutable without mutagenesis. What is curious is that the phenotype of the suppressor plant shows a Ler ‘plant type’ phenotype.

Looking back at the history of the vtc1 mutant, the only time when the Ler and Col genomes were together, described in Conklin et al (1996), was when the vtc1 mutant was crossed to Ler for mapping. The description of the vtc1-1 (soz7) mutant stock (in TAIR) is given as result of 2 x backcrosses and an F3, presumably as a result of crossing to Col-0, but it might be useful to confirm that the stock has no Ler background and the seed used was progeny of single plant and not from a bulk seed lot. Since the VTC1 locus has also been characterized by cyt1 and emb101 mutants, it would seem that the mutant alleles might have some disadvantage in being propagated and a ‘residual heterozygosity’ might persist by some mechanism. In addition, reversion to a wild-type phenotype svt2 might be facilitated by a selection of vigorous embryos into maturity.

The sequence changes in the vtc1-1 and svt2 suggests an origin of a ‘template’ independent of Ler and Col alleles, and might also be sequences from another related ecotype. A screen of available Arabidopsis ecotype genome sequences should show such an alternate donor.

Minor comment: On the PDF, page 10, need to use ‘were’ instead of ‘where’ in the sentence beginning “In all cases...”
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.