RESEARCH ARTICLE

The novel *Arabidopsis thaliana* *svt2* suppressor of the ascorbic acid-deficient mutant *vtc1-1* exhibits phenotypic and genotypic instability [version 1; peer review: 2 approved, 1 not approved]

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V1 First published: 10 Jan 2013, 2:6
<https://doi.org/10.12688/f1000research.2-6.v1>
 Latest published: 10 Jan 2013, 2:6
<https://doi.org/10.12688/f1000research.2-6.v1>

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 *vtc1-1* 2, *svt2*, with wild-type levels of ascorbic acid and root growth similar to the wild type in the presence of ammonium was isolated. Interestingly, *svt2* has *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

Open Peer Review

Approval Status

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version 1
10 Jan 2013	view	view	view

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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

Hopkins MT, Khalid AM, Chang PC et al. » De novo genetic variation revealed in somatic sectors of single Arabidopsis plants, F1000Research 2013, 2:5 (<https://doi.org/10.12688/f1000research.2-5.v2>)

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Competing interests: No relevant competing interests disclosed.

Grant information: This work was supported by a start-up grant of West Virginia University to CB.

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

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How to cite this article: Kempinski CF, Crowell SV, Smeeth C and Barth C. **The novel *Arabidopsis thaliana* svt2 suppressor of the ascorbic acid-deficient mutant vtc1-1 exhibits phenotypic and genotypic instability [version 1; peer review: 2 approved, 1 not approved]** F1000Research 2013, 2:6 <https://doi.org/10.12688/f1000research.2-6.v1>

First published: 10 Jan 2013, 2:6 <https://doi.org/10.12688/f1000research.2-6.v1>

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 senescence¹⁻³, pathogen disease resistance^{2,4}, the biosynthesis of various plant hormones⁵⁻⁷, and root development⁸⁻¹¹. 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/L-galactose pathway^{12,13}. 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 seed¹⁴. The *vtc1-1* mutant contains a point mutation in amino acid 22 that converts a conserved proline into a serine¹⁵. 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 deficiency⁸⁻¹¹. 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 M₀ generation, *svt2* (*suppressor of vtc1-1 2*), 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 M₂ generation produced progeny that exhibited phenotypic and genotypic *svt2* characteristics again in the M₃ generation.

Phenotypic instability of *Arabidopsis* alleles affecting a disease resistance gene cluster has recently been reported¹⁶. 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 mechanism¹⁷.

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 phenome 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* mutant¹⁴ (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⁻² (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 described¹¹. 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 day and night, and 160 μmol photons m⁻² s⁻¹ in a growth chamber (Percival Scientific, Inc., Perry, IA). Each plate contained wild-type and mutant seed. Primary root length was measured in seven-day-old seedlings using a ruler.

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°C¹⁸.

Ethyl methanesulfonate mutagenesis

Seeds of homozygous *vtc1-1 Arabidopsis thaliana* (Col-0 background) were mutagenized with 0.2% ethyl methanesulfonate as described (Figure 1;¹⁸). 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 assay¹⁹. 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* M₂ 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 *svf2*. 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 (M_0) were planted on soil and screened for wild-type levels of ascorbic acid. The only mutant isolated in the M_0 generation containing recovered ascorbic acid levels was *svf2*. 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 protocol³. In case of genomic DNA isolation from *vtc1-1* seeds, a small amount of dried seeds was crushed and the extraction procedure described previously³ 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 μ L. 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 (C_T) were calculated using iQ software (Bio-Rad).

Primer efficiencies (E) were calculated using cDNAs synthesized from RNA isolated from Col-0 plants as previously described¹¹. 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 C_T 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: $E = 10^{(1/\text{slope})}$.

Copy number of *VTC1* (AT2G39770) was calculated using the formula: Reported Quantity (RQ) = $1/E^{CT}$ normalized to the RQ of a known single copy gene (*PAD4*, AT3G52430;^{20,21}). *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

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

Primer Name	5' 3'
<i>VTC1</i> G1 F	AAA AAT TCG TTC TAG ATG GAT GCT
<i>VTC1</i> G1 R	ATG GCT GTA AAT TGG AAG AGA T
<i>VTC1</i> G2 F	GAA CCC TTG TCT CTA AAA TA
<i>VTC1</i> G2 R	CAA ATC CCA TAA TCT GTT CC
<i>VTC1</i> G3 F	CAA TTT TGC TTA CTT CTC T
<i>VTC1</i> G3 R	TGG ATG CAA CCG ACA CAA AAC AAT
<i>VTC1</i> G4 F	ACA TTT TTA GCA GCT GGT ATT GAG
<i>VTC1</i> G4 R	AGG TAA GAA CTG GCA GAC TAA AG
<i>VTC1</i> G5 F	TCG CTT GAG ACC ATT GAC T
<i>VTC1</i> G5 R	GAG GCT TCC CCA CCG TGA GAT TTG
<i>VTC1</i> G6 F	CAA GCT GGA AAT CAA AAT CAC T
<i>VTC1</i> G6 R	GCG CTG CTG CAA TCT TAG G
<i>VTC1</i> G7 F	ACA AAT CTC ACG GTG GGG AAG C
<i>VTC1</i> G7 R	TGG TTA ATT TGG CAG GAG A
<i>VTC1</i> G8 F	CAA GGG CTC TAT GCT ATG GTG
<i>VTC1</i> G8 R	GCG TTT TGA TTG ATG CTT ATT C
<i>VTC1</i> G9 F	GCG TGT ATC TCG AGC AGT ATC AT
<i>VTC1</i> G9 R	GTG GAG GGA AGT TAA GGG TAT TTT
InDel 1 450919 F	ATC GGT TTG TAA TCT CTG TCC A
InDel 1 450919 R	TAT GCG TTC CCA AAT TTG TTA TCT C
InDel 2 451470 F	GGA GAC CCA AAC TGC TAT TAC A
InDel 2 451470 R	AAC CGC CTC CAT TTG CAC CTT ATC
InDel 3 469762 F	GTC ACC GAG TTT TGC TTT GTT CAT
InDel 3 469762 R	CTC GTT TCT TTT CTG GGC TTG TAG
InDel 4 449053 F	GAA AGA AAG CAG CGA AAG ACA
InDel 4 449053 R	GCC CAT GCC CAT ACA CTG A
InDel 5 455100 F	ACT TGC TTA ATC GTT TCT TTG TA
InDel 5 455100 R	GCC CAC TCG TAT TCG CTT AG

three replicates of each respective genotype, all run in the same reaction plate.

Sequencing 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 described¹⁹. The AA content was determined in whole rosettes of three-week-old plants using the iron reduction assay⁴.

Statistical analysis

Experiments were performed at least three times. Figures represent individual experiments. Data were expressed as mean values $\pm$ SE. *P* values were determined by Student's *t* test analysis.

Results

Isolation of *svt2*

Our laboratory is interested in understanding how the *VTC1* gene, which is essential for the biosynthesis of GDP-mannose and AA, is regulated. This would help deciphering the pleiotropic phenotypes displayed by *vtc1-1*, including its hypersensitivity to ammonium^{8–11}. We employed a gene suppressor analysis with the goal of identifying novel genes that interact or regulate *VTC1*. Seed of the *vtc1-1* mutant, which is in the Col-0 genetic background¹⁴, were subjected to chemical mutagenesis using EMS¹⁸. One thousand and one hundred mutagenized *vtc1-1* seeds (M_0 generation) were planted onto soil and screened for recovered (wild-type) leaf AA content using the qualitative nitroblue tetrazolium test¹⁹. One of the mutants exhibited wild-type AA levels in the M_0 generation. This mutant was named *svt2* (suppressor of *vtc1-1* 2), isolated, and further characterized. The mutant was allowed to self-fertilize and seeds from the plant were collected (M_1 generation) (Figure 1). Note that we isolated additional suppressor mutants by pooling M_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. M_3 seed were collected and screened for long roots again to test for segregation. M_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 *vtc1-1* was only approximately 40% of that of the wild type^{14,15}.

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

Root lengths of Col-0 WT, *vtc1-1*, and D3 suppressor mutants (mm)

5 Data Files

<http://dx.doi.org/10.6084/m9.figshare.103765>

Total ascorbic acid of Col-0 WT, *vtc1-1*, and D3 suppressor mutants (μmol gFW⁻¹)

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.103766>

svt2 has similarities with the *Ler* phenotype, but has also phenotypic characteristics that are distinct from *Ler*

The first observation we made when characterizing *svt2* M_1 plants was that *svt2* exhibited a phenotype reminiscent of the *Ler* ecotype with the characteristic round leaves and erect morphology when compared to Col (Figure 3A). However, *svt2* also had features that were distinct from the *Ler* phenotype, including overall enlarged vegetative and reproductive morphology (insets of rosettes and flowers in Figure 3A). In addition, *svt2* was strongly delayed in flowering compared to the Col-0 and *Ler*-0 wild types and the *vtc1-1* mutant (Figure 3A, 3B). Primary inflorescences in four-week-old plants were 1.4-times significantly longer in the *vtc1-1* mutant and approximately twice as

Figure 2. Phenotypic and genotypic characterization of additional *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 *vtc1-1*) or 24 individual replicates. *** $P < 0.001$ by Student's *t*-test indicates significant differences in comparison to the Col-0 wild type. (C) Sequences of the Col-0 wild type, the *vtc1-1* mutant and four suppressor mutants. The arrow points to the *vtc1-1* mutation, a conversion of cytosine to a thymine.

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 previously³.

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^{14,15} (Figure 3C). Finally, we investigated whether *svt2* also exhibits recovered root development in the presence of ammonium by growing the four genotypes in full strength 1× MS medium. The *vtc1-1* mutant is conditionally hypersensitive to ammonium^{8,9,11}. Figure 3D illustrates that root length in *svt2* was the same as in Col-0 wild type, whereas root development was strongly inhibited in *vtc1-1* as expected.

The enlarged morphology of *svt2* raises the question as to whether *svt2* is polyploid. In order to test this, we assessed the size of pollen grains from the Col-0 and *Ler*-0 wild-types and *vtc1-1* and *svt2* mutants. As is shown in Figure 4, pollen grains of the four genotypes are similar in size. In addition, using qPCR, we determined the number of *VTC1* gene copies in the four genotypes. Our results revealed that *VTC1* is present as a single copy gene in both the Col-0 and *Ler*-0 wild types and in the *vtc1-1* and *svt2* mutants (Table 2). Although an extensive chromosome analysis has not yet been performed in *svt2*, our results suggest that the mutant does not contain additional sets of chromosomes.

Taken together, based on the phenotypic observations, our data suggest that *svt2* represents a novel *vtc1-1* suppressor mutant with recovered AA content and root development. Next, we characterized *svt2* genotypically in order to determine whether *svt2* represents an intragenic or extragenic suppressor.

svt2 shares genome similarity with *Ler*

To determine whether *svt2* represents an intragenic suppressor, i.e., to test whether the suppressor mutation is present within the *VTC1* gene, we designed nine overlapping PCR primer sets spanning the entire *VTC1* gene and approximately 500 bp of the promoter region directly upstream of the first base in the 5' UTR (Table 1, Figure 5A). PCR products were generated from genomic DNA extracted from Col-0 and *Ler*-0 wild types, and *vtc1-1* and *svt2* mutants. In eight of the nine primer pairs covering the entire *VTC1* gene, the PCR products generated using *svt2* genomic DNA had the same electrophoretic mobility as those generated using Col-0 wild-type genomic DNA. However, this was not the case for the first primer set. The G1F/G1R primer set, used to amplify the *VTC1* promoter region, generated a larger PCR product in *svt2* than in the wild type (Figure 5B, Figure 6). The PCR product in the wild type was 567 bp, whereas that in *svt2* had a size of approximately 850 bp, suggesting that *svt2* contained an approximately 300 bp insertion in this region. We repeated the PCR analysis of the *VTC1* promoter region using the G1F-G1R and the G1F-G2R primer sets that should generate a PCR product of 567 bp and 751 bp, respectively (Figure 5A). The expected size was obtained for the Col-0 wild type and the *vtc1-1* mutant. However, approximately 300 bp larger PCR products were detected in the *svt2* mutant and the *Ler*-0 wild type (Figure 5B),

suggesting a *Ler* insertion polymorphism. Thus, these data imply that *svt2* shares both phenotypic and genotypic similarities with *Ler*.

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 InDels 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 1990); 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*.

Quantitative PCR to verify that *VTC1* is a single copy gene in Col-0 and *Ler*-0 wild types and *vtc1-1* and *svt2* mutants

2 Data Files

<http://dx.doi.org/10.6084/m9.figshare.103771>

svt2 exhibits phenotypic and genotypic instability

Our initial observations revealed that approximately 10% of *svt2* M₂ 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 $\pm$ 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 $\pm$ SE of three individual replicates. (D) Root length in seven-day-old seedlings grown on 1 $\times$ MS. Bars represent means $\pm$ 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.

Primary inflorescence length of Col-0 WT, vtc1-1, svt2 (M1) and Ler-0 WT (cm)

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.103767>

Total ascorbic acid of Col-0 WT, vtc1-1, svt2 (M1) and Ler-0 WT (μmol gFW-1)

3 Data Files

<http://dx.doi.org/10.6084/m9.figshare.103768>

Root lengths of Col-0 WT, vtc1-1, svt2 (M2) and Ler-0 WT (mm)

3 Data Files

<http://dx.doi.org/10.6084/m9.figshare.103769>

Figure 4. Pollen phenotype of Col-0 wild type, the *vtc1-1* and *svt2* mutants and the *Ler-0* wild type. Light images were taken when plants were 4.5 weeks old. Scale bar represents 10 μm.

Table 2. Quantitative PCR to verify that *VTC1* is a single copy gene in Col-0 and *Ler-0* wild types and *vtc1-1* and *svt2* mutants. Quantitative PCR was performed as described in Materials and Methods. The *PAD4* gene is a known single copy gene. Therefore, an RQ/RQ ratio of approximately 1 indicates that *VTC1* is present in similar quantity as *PAD4*, and therefore a single-copy gene.

	RQ/RQ	
	<i>VTC1/PAD4</i>	
Col-0 WT	0.3796	
<i>vtc1-1</i>	0.5843	VTC1 E=1.8
<i>svt2</i>	0.5504	
<i>Ler-0</i> WT	0.6329	
Col-0	0.3153	
<i>vtc1-1</i>	0.5292	VTC1 E=2
<i>svt2</i>	0.4946	
<i>Ler-0</i> WT	0.5807	

we investigated whether *svt2* progeny that were phenotypically Col-like revertants would produce *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 *svt2* M₁ generation, only 1% of Col-like revertants were detected. In contrast, 8–10% of *svt2* M₂ plants displayed a Col-like phenotype, whereas no revertants were detected in the *svt2* M₃ generation. These Col-like revertants were isolated and seeds were collected from individual plants and the phenotype of the progeny in the M₃ generation was assessed in some examples. In most cases, reversion appeared to be stable, i.e., once *svt2* plants reverted, displaying a Col-like phenotype in the M₂ generation, their M₃ progeny continued to appear as Col-like plants. This was the case for the M₃ 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 *svt2*-like phenotype (Table 3), i.e., the K1 double revertant switched from *svt2* phenotype in the M₁ generation to a Col-like phenotype in the M₂ generation, and then reverted back to a *svt2*-like phenotype in the M₃ generation. Note that only a small number of progeny was planted. In a second experiment, the *svt2* Col R1 revertant produced 20 individuals displaying a *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 *svt2* → Col single revertants (Col R1, Col R2, K1 Col R) and a *svt2* → Col → *svt2* double revertant (K1 Col R *svt2* R).

Next we tested whether a Col-like revertant phenotype correlated with a Col-like genotype. Likewise, we would expect that a *svt2* → Col → *svt2* double revertant phenotype corresponds with *svt2*-like genomic markers. To check this we isolated genomic DNA from Col-0 and *Ler-0* wild types, *svt2*, *vtc1-1* and revertant mutants, and PCR-amplified the five randomly selected InDel polymorphisms plus the InDel polymorphism in the *VTC1* promoter (Table 1). In all cases but the *svt2* M₂ Col R1 revertant, the *svt2*-like revertant plants (labeled *svt2* M₂ 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, *svt2* M₁ plants and *svt2* M₂ plants that displayed an *svt2* phenotype, gave rise to PCR products that were of the same electrophoretic mobility as those of the *Ler* wild type (Table 4, Figure 9). In addition, the double revertant plant K1 (labeled *svt2* M₂ K1 Col R) was genotyped in both its M₂ and M₃ generations. The K1 plant produced InDel PCR products similar to those of the Col-0 wild type in the M₂ generation. However, the M₃ generation that displayed *svt2*-like morphology produced PCR products that were comparable to the InDel PCR products generated using *Ler* genomic DNA (Table 4). The *svt2* M₂ Col R1 (highlighted in red in Table 4) is intriguing, because it appears to contain DNA that is similar to both Col and *Ler* genomic DNA. This suggests the presence of chimeric genome sectors, which may explain the higher percentage of Col-like revertants compared to *svt2* M₂ 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.

PCR amplification of the *VTC1* promoter region in the Col-0 wild type, *vtc1-1* and *svt2* mutants

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.103770>

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.

Figure 7. Insertion/Deletion polymorphism analysis in *svt2*, *Col-0* and *Ler-0*. Primers were designed for five randomly selected InDel polymorphisms across the five *Arabidopsis* chromosomes. The polymorphisms represent insertions in *Col-0* and deletions in *Ler*.

Table 3. Summary of revertant data. The table summarizes the number of plants screened in each of three *svt2* generations (M_1 , M_2 and M_3), 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	<i>svt2</i> M_1	63	0	0
	<i>svt2</i> M_2 , 3 of 7 revertants tested further:		7 (Col phenotype)	8.97
	<i>svt2</i> A8 Col R M_3	78	0	0
	<i>svt2</i> G7 Col R M_3	64	0	0
	<i>svt2</i> K1 Col R M_3 *	64	1 (<i>svt2</i> phenotype)	1.58
	<i>svt2</i> K1 Col R <i>svt2</i> R M_4 *	63	0	0
		96		
2	<i>svt2</i> M_3	96	0	0
	<i>svt2</i> M_1	96	1 (Col phenotype)	1.04
	<i>svt2</i> M_2 , 2 of 5 revertants tested further:	62	5 (Col phenotype)	8.06
	<i>svt2</i> Col R1 M_3 *	88	20 (<i>svt2</i> phenotype)	22.73
	<i>svt2</i> Col R4 M_3 *	96	0	0
3	<i>svt2</i> M_2	96	10 (Col phenotype)	10.42

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

Figure 8. Phenotype of *svt2* revertants. Plants were three weeks old when photographs were taken. Top row represents controls, Col-0 wild type, *vtc1-1* and *svt2* mutants, and *Ler-0* wild type. Bottom row represents three Col-like revertants, *svt2* Col R1 M₃, *svt2* Col R2 M₃, *svt2* K1 Col R M₃, and a double revertant, *svt2* K1 Col R *svt2* R M₄. R stands for revertant.

Table 4. Summary of PCR-based molecular genotypes. With the exception of *svt2* Col R1 M₂, where Col and 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 Col polymorphisms, while a Ler-like phenotype correlated with Ler polymorphisms. C, L, and H refer to Col, Ler, or heterozygous, respectively. R denotes revertant. n.d., not detected.

Genotype	InDel 1 450919	InDel 2 451470	InDel 3 469762	InDel 4 449053	InDel 5 455100	G1F + G2R <i>VTC1</i>
Col-0 WT	C	C	C	C	C	C
<i>vtc1-1</i>	C	C	C	C	C	C
<i>Ler-0</i> WT	L	L	L	L	L	L
<i>svt2</i> M ₁	L	L	L	L	L	L
<i>svt2</i> M ₂	L	L	L	L	L	L
<i>svt2</i> Col R1 M ₂	C	L	C	H	C	C
<i>svt2</i> Col R2 M ₂	C	C	C	C	C	C
<i>svt2</i> Col R3 M ₂	C	C	C	C	n.d.	C
<i>svt2</i> Col R4 M ₂	C	C	C	C	C	C
<i>svt2</i> Col R5 M ₂	C	C	C	C	C	C
<i>svt2</i> K1 Col R M ₂	C	C	C	C	C	C
<i>svt2</i> K1 Col R <i>svt2</i> R M ₃	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>

from one generation to the next, termed transgenerational epigenetic inheritance^{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 *svt2* donor should have a *svt2*-like phenotype. To determine the genotypes of the F₁ progeny of the reciprocal crosses, we performed another InDel polymorphism assay as described above. In addition, progeny were also screened using the *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 Col and *Ler* genomic DNA, respectively, were generated, suggesting that the F₁ of the reciprocal crosses were heterozygous. A similar result was obtained for the *VTC1* promoter polymorphism marker in all reciprocal crosses. Note, however, that for some polymorphisms and irrespective of whether *svt2* or Col-0 served as female or male donor, respectively, PCR products comparable to those obtained using *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 *svt2*. However, at some loci *svt2*-like alleles dominate over Col-0.

Discussion

The *svt2* mutant was initially identified as a putative suppressor of the AA-deficient *Arabidopsis* mutant *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, *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.

svt2 is a novel *Arabidopsis* mutant and not a result of an experimental artifact, seed contamination, or outcrossing

Several lines of evidence support our findings that *svt2* is a novel mutant. First, *svt2* was the only suppressor mutant isolated among over 1000 EMS-mutagenized M₀ 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 *VTC1* promoter, and additional SNPs (Figure 5B–D, Figure 6, Figure S1). These data demonstrate that *svt2* has acquired new characteristics, presumably as a result of EMS mutagenesis, and that *svt2* is neither Col nor *Ler*. These data also argue against *svt2* being an experimental or PCR artifact.

Second, a number of data provide strong arguments against seed contamination. (1) With high reproducibility, descendants of the original *svt2* mutant produce offspring revertants with Col-like features (Table 3, Table 4; Figure 8, Figure 9). (2) One of the Col-like revertants, *svt2* Col R1 M₃, exhibited heterozygosity at some of the InDels tested (Table 4). (3) One of those Col-like revertants, *svt2* K1 Col R M₃, produced progeny that reverted

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 × Male crosses	InDel 1 450919	InDel 2 451470	InDel 3 469762	InDel 4 449053	InDel 5 455100	G1F + G2R VTC1
<i>svt2</i> × Col-0 F ₁ 1	H	H	L	H	H	H
<i>svt2</i> × Col-0 F ₁ 2	H	H	L	H	H	H
<i>svt2</i> × Col-0 F ₁ 3	H	H	H	H	H	H
<i>svt2</i> × Col-0 F ₁ 4	H	H	H	H	H	H
Col-0 × <i>svt2</i> F ₁ 1	H	H	H	L	H	H
Col-0 × <i>svt2</i> F ₁ 2	H	H	L	H	H	H
Col-0 × <i>svt2</i> F ₁ 3	H	H	H	L	H	H
Col-0 × <i>svt2</i> F ₁ 4	H	H	L	H	H	H

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

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.103774>

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>

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

initial planting of the *vtc1-1* M₀ 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 polyploidy²⁴. 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 polyploids²⁵. 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 heterozygosity²⁵, low fertility, and low embryonic viability^{26–28}. This, however, is not the case in *svt2*. The fact that *svt2* is fertile and that its enlarged morphology is heritable 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 levels²⁹.

Instead, we hypothesize that genome instability of *svt2* was further aggravated by exposing the already instable 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 breaks³⁰. 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*³⁰. 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^{31,32}. In the process of soybean cell culture, massive specific changes in numerous genome-wide loci were observed³¹. 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 conditions³².

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 damage³³. 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 screen¹⁵. 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 pools¹⁵. 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 lethal³⁴. To date, only the *vtc1-1*¹⁵ and *hsn1* mutations⁸ 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 in which 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 γ -radiation has been reported to induce high frequency phenotypic instability in the *Arabidopsis* disease resistance genes *CPR1* and *BAL*, which map to the *RPP5* locus¹⁶. Yi and Richards reported destabilization of phenotypes in both the *bal* and *cpr1* mutants in more than 10% of EMS-treated plants in the M₁ generation. They also identified exceptions to simple Mendelian inheritance in the M₂ generation. Phenotypic instability was also observed in *bal* $\times$ *cpr1* F₁ 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 *SNCl* gene¹⁷.

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* mutant³⁷. 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 reported³⁸. Lolle *et al.* postulated that these genetic restoration events are the result of a template-directed process that utilizes

an ancestral RNA-sequence cache³⁷. This hypothesis is supported by observations reported by Xu and co-workers³⁸. 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

This work was supported by a start-up grant of West Virginia University to CB.

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

Acknowledgements

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.

Supplementary material

[illegible]

	2710	2720	2730	2740	2750	2760
						
Col-0_VTC1_TAIR_gDNA	ACTTGCCATATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCTGACGC					
vtc1-1_VTC1_gDNA	ACTTGCCATATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCTGACGC					
svt2_VTC1_gDNA	ACTTGCCATATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCTGACGC					
Ler-0_VTC1_GenBank_gDNA	ACTTGCCATATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCT~ACGC					
	2770	2780	2790	2800	2810	2820
						
Col-0_VTC1_TAIR_gDNA	AACCGCTCAGGCTGATCTCTTCCAATTTACAGCCATTTCCCAGCTCAGATCTCTGATCCG					
vtc1-1_VTC1_gDNA	AACCGCTCAGGCTGATCTCTTCCAATTTACAGCCATTTCCCAGCTCAGATCTCTGATCCG					
svt2_VTC1_gDNA	AACCGCTCAGGCTGATCTCTTCCAATTTACAGCCATTTCCCAGCTCAGATCTCTGATCCG					
Ler-0_VTC1_GenBank_gDNA	AACCGCTCAGGCTGATCTCTTCCAATTTACAGCCATTTCCCAGCTCAGATCTCTGATCCG					
	2830	2840	2850	2860	2870	2880
						
Col-0_VTC1_TAIR_gDNA	GTGAGATCTCTCTCAAGGTAATGCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG					
vtc1-1_VTC1_gDNA	GTGAGATCTCTCTCAAGGTAATGCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG					
svt2_VTC1_gDNA	GTGAGATCTCTCTCAAGGTAATGCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG					
Ler-0_VTC1_GenBank_gDNA	GTGAGATCTCTCTCAAGGTAATGCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG					
	2890	2900	2910	2920	2930	2940
						
Col-0_VTC1_TAIR_gDNA	CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCATTTTGTATTTGCTGTTGTT					
vtc1-1_VTC1_gDNA	CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCATTTTGTATTTGCTGTTGTT					
svt2_VTC1_gDNA	CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCATTTTGTATTTGCTGTTGTT					
Ler-0_VTC1_GenBank_gDNA	CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCATTTTGTATTT~CTGTTGTT					
	2950	2960	2970	2980	2990	3000
						
Col-0_VTC1_TAIR_gDNA	GGTTTTTAATTCGATTTTCCGGAACAGATTATGGGATTT~GTATTCGAATCTTCGATTTG					
vtc1-1_VTC1_gDNA	GGTTTTTAATTCGATTTTCCGGAACAGATTATGGGATTT~GTATTCGAATCTTCGATTTG					
svt2_VTC1_gDNA	GGTTTTTAATTCGATTTTCCGGAACAGATTATGGGATTTGTATTCGAATCTTCGATTTG					
Ler-0_VTC1_GenBank_gDNA	GGTTTTTAATTCGATTTTCCGGAACAGATTATGGGGATTTGTAT~CGAATCTTCGATTTG					
	3010	3020	3030	3040	3050	3060
						
Col-0_VTC1_TAIR_gDNA	ATGACATAATGTCCCAGCCTTTTATGTTAATCTTGAAATGATGGACTTTTATCCGATCT					
vtc1-1_VTC1_gDNA	ATGACATAATGTCCCAGCCTTTTATGTTAATCTTGAAATGATGGACTTTTATCCGATCT					
svt2_VTC1_gDNA	ATGACATAATGTCCCAGCCTTTTATGTTAATCTTGAAATGATGGACTTTAATCCGATCT					
Ler-0_VTC1_GenBank_gDNA	ATGACATAATGTCCCAGCCTTTTATGTTAATCTTGAAATGATGGACTTTAATCCGATCT					
	3070	3080	3090	3100	3110	3120
						
Col-0_VTC1_TAIR_gDNA	GGGTTTAAAGCTGGAATTTTGATTGTGGGTACTATTAGGTTTCATTGATTTATTGCTTGG					
vtc1-1_VTC1_gDNA	GGGTTTAAAGCTGGAATTTTGATTGTGGGTACTATTAGGTTTCATTGATTTATTGCTTGG					
svt2_VTC1_gDNA	GGGTTTAAAGCTGGAATTTTGATTGTGGGTACTATTAGGTTTCATTGATTTATTGCTTGG					
Ler-0_VTC1_GenBank_gDNA	GGGTTTAAAGCTGGAATTTTGATTGTGGGTACTATTAGGTTTCATTGATTTATTGCTTGG					
	3130	3140	3150	3160	3170	3180
						
Col-0_VTC1_TAIR_gDNA	TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGCTGAATTTTGGAAAGAACTATT					
vtc1-1_VTC1_gDNA	TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGCTGAATTTTGGAAAGAACTATT					
svt2_VTC1_gDNA	TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGCTGAATTTTGGAAAGAACTATT					
Ler-0_VTC1_GenBank_gDNA	TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGCTGAATTTTGGAAAGAACTATT					
	3190	3200	3210	3220	3230	3240
						
Col-0_VTC1_TAIR_gDNA	TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTACCTTTTTCTCTGATTATT					
vtc1-1_VTC1_gDNA	TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTACCTTTTTCTCTGATTATT					
svt2_VTC1_gDNA	TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTACCTTTTTCTCTGATTATT					
Ler-0_VTC1_GenBank_gDNA	TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTACCTTTTTCTCTGATTATT					

	3250 3260 3270 3280 3290 3300
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	GTTTGTGTCGGTTGCATCCACTTTGATTAGATCTGAATGAATCATTTTTTATGTGCTC
svt2_VTC1_gDNA	GTTTGTGTCGGTTGCATCCACTTTGATTAGATCTGAATGAATCATTTTTTATGTGCTC
Ler-0_VTC1_GenBank_gDNA	GTTTGTGTCGGTTGCATCCACTTTGATTAGATCTGAATGAATCATTTTTTATGTGCTC
	3310 3320 3330 3340 3350 3360
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	AAGTTATTGTATGGATTGTTCTGTTTCTAGCATGTTTGGTTAGACATTGTTAAGATCTG
svt2_VTC1_gDNA	AAGTTATTGTATGGATTGTTCTGTTTCTAGCATGTTTGGTTAGACATTGTTAAGATCTG
Ler-0_VTC1_GenBank_gDNA	AAGTTATTGTATGGATTGTTCTGTTTCTAGCATGTTTGGTTAGACATTGTTAAGATCTG
	3370 3380 3390 3400 3410 3420
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	ACGTTTGCAATTTTCAGGAAAAGGAG~TTAGAGCATCATCAAGATGAAGGCACCTATTCTT
svt2_VTC1_gDNA	ACGTTTGCAATTTTCAGGAAAAGGAG~TTAGAGCATCATCAAGATGAAGGCACCTATTCTT
Ler-0_VTC1_GenBank_gDNA	ACGTTTGCAATTTTCAGGAAAAGGAGCTTAGAGCATCATCAAGATGAAGGCACCTATTCTT
	3430 3440 3450 3460 3470 3480
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	GTTGGAGGCTTCGGCACTCGCTTGAGACCATTGACTCTCAGTTTCCAAAGCCCCTTGTT
svt2_VTC1_gDNA	GTTGGAGGCTTCGGCACTCGCTTGAGACCATTGACTCTCAGTTTCCAAAGCCCCTTGTT
Ler-0_VTC1_GenBank_gDNA	GTTGGAGGCTTCGGCACTCGCTTGAGACCATTGACTCTCAGTTTCCAAAGCCCCTTGTT
	3490 3500 3510 3520 3530 3540
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	GATTTTGCTAATAAACCCATGATCCTTCATCAGGTAATCTATCTTAAATTTGCCGCTTTA
svt2_VTC1_gDNA	GATTTTGCTAATAAACCCATGATCCTTCATCAGGTAATCTATCTTAAATTTGCCGCTTTA
Ler-0_VTC1_GenBank_gDNA	GATTTTGCTAATAAACCCATGATCCTTCATCAGGTAATCTATCTTAAATTTGCCGCTTTA
	3550 3560 3570 3580 3590 3600
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	GCTGCCAGTTCCTTACCTATGCCTATGTTTGAACCGAGGCATGTTTTCTTGTAGATAGAG
svt2_VTC1_gDNA	GCTGCCAGTTCCTTACCTATGCCTATGTTTGAACCGAGGCATGTTTTCTTGTAGATAGAG
Ler-0_VTC1_GenBank_gDNA	GCTGCCAGTTCCTTACCTATGCCTATGTTTGAACCGAGGCATGTTTTCTTGTAGATAGAG
	3610 3620 3630 3640 3650 3660
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	GCTCTTAAGGCAGTTGGAGTTGATGAAGTGGTTTTGGCCATCAATTATCAGCCAGAGGTA
svt2_VTC1_gDNA	GCTCTTAAGGCAGTTGGAGTTGATGAAGTGGTTTTGGCCATCAATTATCAGCCAGAGGTA
Ler-0_VTC1_GenBank_gDNA	GCTCTTAAGGCAGTTGGAGTTGATGAAGTGGTTTTGGCCATCAATTATCAGCCAGAGGTA
	3670 3680 3690 3700 3710 3720
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	AGATACTAATCTCTCTTAACCTTTTTTTTTTGCAGCTATTTTCTGTTTACATATGTTTGTA
svt2_VTC1_gDNA	AGATACTAATCTCTCTTAACCTTTTTTTTTTGCAGCTATTTTCTGTTTACATATGTTTGTA
Ler-0_VTC1_GenBank_gDNA	AGATACTAATCTCTCTTAACCTTTTTTTTTTGCAGCTATTTTCTGTTTACATATGTTTGTA
	3730 3740 3750 3760 3770 3780
Col-0_VTC1_TAIR_gDNA	
vtc1-1_VTC1_gDNA	TTTACCATTGCTCTGTTTCGACAGGTGATGCTGAACCTCTTGAAGGACTTTGAGACCAA
svt2_VTC1_gDNA	TTTACCATTGCTCTGTTTCGACAGGTGATGCTGAACCTCTTGAAGGACTTTGAGACCAA
Ler-0_VTC1_GenBank_gDNA	TTTACCATTGCTCTGTTTCGACAGGTGATGCTGAACCTCTTGAAGGACTTTGAGACCAA

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.

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2 K1 Col R M3_G1F
svt2 K1 Col R svt2 R M4_G1F

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2 K1 Col R M3_G1F
svt2 K1 Col R svt2 R M4_G1F

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2_K1_Col_R_M3_G1F
svt2_K1_Col_R_svt2_R_M4_G1F

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2 K1 Col R M3_G1F
svt2 K1 Col R svt2 R M4_G1F

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2_K1_Col R M3_G1F
svt2_K1_Col R svt2_R M4_G1F

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2_K1_Col R M3_G1F
svt2_K1_Col R svt2_R M4_G1F

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2 K1 Col R M3 G1F
svt2 K1 Col R svt2 R M4 G1F

Col-0_VTC1_gDNA_TAIR
Ler-0_VTC1_gDNA_Genbank
svt2 K1 Col R M3 G1F
svt2 K1 Col R svt2 R M4 G1F

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Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2210      2220      2230      2240      2250
      ....|....|....|....|....|....|....|....|....|....|
      ACCACTACACATTCTTCATACAGCTGAGTTAGGAATGTTAACAAAATAGT
      ACCACTACACATTCTTCATACAGCTGAGTTAGGAATGTTAACAAAATAGT
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2260      2270      2280      2290      2300
      ....|....|....|....|....|....|....|....|....|....|
      ACATAATACCATCCCTTTAAAAACACAGAAT
      TTATGGGAGTATGTTTTATACATAATACCATCCCTTTAAAAACACAGAAT
      CATAATACCATCCCTTTAAAAA-CACAGAAT
      TTATGGGAGTATGTTTTATACATAATACCATCCCTTTAAAAACACAGAAT
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2310      2320      2330      2340      2350
      ....|....|....|....|....|....|....|....|....|....|
      TTTTTTTATCATCTCTGAAACAAATCATTACAGTAGTAAATGTCAA--C
      TTCTTTTATCATCTCCGAAACAAATCATTACAGTAGTAAATGTAAAAAC
      TTTTCTTTATCATCTCTGAAACAAATCATTACAGTAGTAAATGTCAA--C
      TTCTTTTATCATCTCCGAAACAAATCATTACAGTAGTAAATGTAAAAAC
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2360      2370      2380      2390      2400
      ....|....|....|....|....|....|....|....|....|....|
      ACAACATTAAATCTGTTT-GTTGTTGGCATTACAAATGCAAAATCATTT
      ACAACATTAAATCTGTTT-GTTGTTGGCATTACAAATGCAAAATCATTT
      ACAACATTAAATTTGGTTTGTGTTGGCATTACAAATGCAAAATCATTT
      ACAACATTAAATCTGTTT-GTTGTTGGCATTACAAATGCAAAATCATTT
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2410      2420      2430      2440      2450
      ....|....|....|....|....|....|....|....|....|....|
      TCTCATTTTATTATTCGATTTATTTTGTCAAGAACCCTTGCTCTAAAAT
      TCTCATTTTATTATTCGATTTATTTTGTCAAGAACCCTTGCTCTAAAAT
      TCTCATTTTATTATTCGATTTATTTTGTCAAGAACCCTTGCTCTAAAAT
      TCTCATTTTATTATTCGATTTATTTTGTCAAGAACCCTTGCTCTAAAAT
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2460      2470      2480      2490      2500
      ....|....|....|....|....|....|....|....|....|....|
      ATTCATAGAAAAAGAAAGAGCCATTAATTAAATGGCTTGAAGAAAGA-TT
      ATTCATAGAAAAA-AAAAGAGCCATTAATTAAATGGCTTGAAGAAAGA-TT
      ATTCATAGAAAAAGAAAGAGCCATTAATTAAATGGCTTGAAGAAAGA-TT
      ATTCATAGAAAAA-AAAAGAGCCATTAATTAAATGGCTTGAAGAAAGAATT
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2510      2520      2530      2540      2550
      ....|....|....|....|....|....|....|....|....|....|
      GGTGTATAAGC-GTCTACGTGACCTTTAA--TTAATTTACTT---CCCCC
      GGTGTATAAGC-GTCTACGTGACCTTTAA--TTAATTTACTT---CCCCA
      GGTGTATAAGC-GTCTACGTGACCTTTAA--TTAATTTACTT---CCCCC
      GGTGTATAAACCCTACCTGACCTTTAAATTAATTTACTTTCCCCCA
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2560      2570      2580      2590      2600
      ....|....|....|....|....|....|....|....|....|....|
      AAAAAAGTCAAC-ATTCAA-CATGTG-AATAAAAA-TCAATATTGG-TTT
      AAAAAAGTCAAC-ATTCAA-CATGTG-AATAAAAAATCAATATTGG-TTT
      AAAAAAGTCAAC-ATTCAA-CATGTG-AATAAAAA-TCAATATTGG-TTT
      CAAAAAGTCAACCATTCAAACATGGGGAGTAAAAAATCAATATTGGGTTG
  
```

Col-0_VTC1_gDNA_TAIR
 Ler-0_VTC1_gDNA_Genbank
 svt2 K1 Col R M3_G1F
 svt2 K1 Col R svt2 R M4_G1F

```

      2610      2620      2630      2640      2650
      ....|....|....|....|....|....|....|....|....|....|
      CTAAGTAA-GTAAGTACCATATTATTAAATTATTTATTTT-GGTAAATAC
      CTAAGTAA-GTAAGTACCATATTATTAAATTATTT-ATTTTGGTAAATAC
      CTAAGTAA-GTAAGTACCATATTATTAAATTATTTATTTT-GGTAAATAC
      CTAAGTAAAGTAAAGTACCATATTATTAAATTATTTATTTTGGTAAAGA
  
```

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 revertant (Col-like phenotype) and the svt2 K1 Col R svt2 R M₄ 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 svt2 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.

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Version 1

Reviewer Report 31 January 2013

<https://doi.org/10.5256/f1000research.866.r747>

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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 M_0 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 M_0 , 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 M_0 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 M_1 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 as 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 M_0 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 M_2 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.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and 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.

Reviewer Report 31 January 2013

<https://doi.org/10.5256/f1000research.866.r744>

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Igor Kovalchuk

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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.

Competing Interests: No competing interests were disclosed.

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

Reviewer Report 25 January 2013

<https://doi.org/10.5256/f1000research.866.r730>

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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* (*soz1*) 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..."

Competing Interests: No competing interests were disclosed.

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

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