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Understanding the role of ascorbic acid and GDP-mannose pyrophosphorylase affecting ammonium sensitivity, genome stability and flowering time in *Arabidopsis thaliana*

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Thesis submitted to the
Eberly College of Arts and Sciences
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in
Biology

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ABSTRACT

Understanding the role of ascorbic acid and GDP-mannose pyrophosphorylase affecting ammonium sensitivity, genome stability and flowering time in *Arabidopsis thaliana*

Chase F. Kempinski

L-ascorbic acid is an important antioxidant in both plants and animals. In plants, it is important for detoxifying reactive oxygen species that are produced during photosynthesis and cellular metabolism. It also contributes to several facets of plant growth as an enzyme co-factor, signaling molecule, and a precursor to several other metabolites. It has been implicated in the control of flowering time and senescence as well as several other growth processes largely through work with the ascorbic acid-deficient *vtc* mutants of *Arabidopsis thaliana*. Biochemical and genetic experiments have identified several pathways contributing to ascorbic acid biosynthesis, with the D-mannose/L-galactose pathway predominantly responsible for the accumulation of ascorbic acid in Arabidopsis. Key enzymes in this pathway include GDP-mannose pyrophosphorylase (encoded by *VTC1*), GDP-galactose phosphorylase (encoded by *VTC2/5*), and galactose-1-P phosphatase *VTC4* genes.

Nitrogen is one of the crucial minerals for plant growth and often one of the most limiting in nature. Ammonium is the favored form of nitrogen taken up by plants, but excess levels lead to toxicity since ammonia (the conjugate base) can diffuse across membranes and depolarize membrane potentials. Recent work has indicated that the enzyme GDP-mannose pyrophosphorylase (VTC1 in *Arabidopsis thaliana*), which generates the essential nucleotide sugar GDP-mannose, important for protein N-glycosylation, plays an important role in response to ammonium. *Arabidopsis* mutants with defective VTC1 have stunted growth when grown in tissue culture in the presence of ammonium. We demonstrate here that the response of VTC1 to ammonium is pH-dependent and is not a result of ascorbic acid deficiency and is largely independent of the defects in protein N-glycosylation. We speculate that VTC1 activity is regulated in a pH-dependent manner and discuss our findings in the context of recent reports showing that GDP-mannose pyrophosphorylase forms oligomers necessary for optimal enzyme activity.

Currently, the Mendelian inheritance of genetic information is regarded as a core tenet in our understanding of how genetic information is passed from one generation to the next. However, recent experiments have shown that plants are able to produce progeny that are genetically unique from their parents. The genotypes of these progeny are not predictable given the laws of Mendelian inheritance and a decisive explanation as to how they arise is still not known. Often, these genetically unique progeny are disregarded as experimental errors or contaminants. However, we have isolated a novel *Arabidopsis* mutant, *svt2*, which is capable of producing genetically distinct progeny from self-pollinated plants at a persistent relatively high rate (~10% of progeny exhibit a genotype different from the parent). The *svt2* mutant was

isolated in a suppressor screen of the *vtc1-1* mutant, which aimed to identify genes important for the ammonium sensitivity exhibited by *vtc1-1*. Further characterization of the isolated M_0 plant revealed a genotype that was different from *vtc1-1* and the wild type (Columbia-0 [Col-0] accession), but was more similar to the genotype of Landsberg *erecta*-0 (L*er*-0). Multiple experiments ruled out possible seed or pollen contamination. Furthermore, *svt2* offspring with Col-like characteristics can produce plants with L*er*-like features, suggesting genotypic and phenotypic instability in *svt2*. We speculate that the additive stress of the chemical mutagen used to generate *svt2* and the elevated oxidative stress in the *vtc1-1* mutant, trigged activation of a genome restructuring event that is an inherent capability present in plants. This is supported by other studies which show plants under intense abiotic stresses can produce genotypically different progeny.

The ascorbic acid deficiency in the *vtc* mutants causes several pleiotropic phenotypes beyond oxidative stress, including early flowering and senescence. In a study to identify the flowering pathway that ascorbic acid interacts in and causes this phenotype, a double mutant with a defect in the flowering time gene, *FCA* (*fca-1*, in the L*er*-0 background), and *vtc1-1* had partially recovered ascorbic acid but maintained a delayed flowering time. This suggested interaction of FCA or one of its interacting partners in ascorbic acid biosynthesis. Further analyses indicated that FCA does not act directly affect transcription of genes within the D-mannose/L-galactose pathway, but may act through post-transcriptional regulation. We speculate that the increased ascorbic acid in *vtc1-1 fca-1* is most likely caused by the differing accession backgrounds of the two mutants. However, since *vtc1-1 fca-9* and *vtc4-1 fca-9* mutants (*fca-9* is in the Col-0 background) exhibit significantly increased ascorbic acid compared to their respective single *vtc* mutants, FCA does indeed appear to play a role in ascorbic acid biosynthesis but does so through an unidentified mechanism in the D-mannose/L-galactose pathway or possibly in another biosynthetic pathway.

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1 CHAPTER 1: Toward the mechanism of NH_4^+ sensitivity mediated by *Arabidopsis* GDP-mannose pyrophosphorylase

1.1 INTRODUCTION

1.1.1 Ascorbic acid as an antioxidant and enzyme cofactor in plants

L-ascorbic acid (AA) is an important antioxidant molecule in both plants and animals. Its main function is to detoxify reactive oxygen species (ROS) produced during respiration and photosynthesis (Noctor and Foyer, 1998; Conklin, 2001). In plants, it also functions as a cofactor for violaxanthin de-epoxidase and dioxygenase enzymes, which are important for the synthesis of the phytohormones ethylene and giberrellic acid (GA); hydroxyproline and hydroxylysine, which are important cell wall components (Conklin, 2001) as well as the synthesis of many other secondary metabolites (Arrigoni and De Tullio, 2000; Smirnoff, 2000; Arrigoni and De Tullio, 2002).

1.1.2 Ascorbic acid biosynthesis

Ascorbic acid is the most abundant antioxidant within plant cells with average values ranging from 2-25 mM and even higher amounts in the plastids (Smirnoff, 2000). There are three identified pathways in *Arabidopsis thaliana* that contribute to AA biosynthesis (Fig. 1.1): the L-galactose/D-mannose pathway, initially characterized by Wheeler et al. (1998), which contributes to the majority of the plant AA, the D-galacturonic acid pathway, and the *myo*-inositol pathway (Lorence et al., 2004; Hancock and Viola, 2005). It should be noted that Wolucka and Van Montagu (2003) showed *in vitro* that GDP-mannose 3', 5' epimerase is capable of producing both GDP-L-galactose and GDP-L-gulose. They also demonstrated that plants incorporate L-gulose into AA, indicative of an alternative route to AA that branches from the initially characterized D-mannose/L-galactose pathway. Using biochemical and genetic assays, Wheeler et al. (1998), described the L-galactose/D-mannose pathway (Fig. 1.1) after discovering that *Arabidopsis* plants fed with L-galactose had an increased AA content similar to plants fed with L-galactono-1,4-lactone. This provided evidence to demonstrate that plants can synthesize AA without inversion of the hexose carbon skeleton, which is known not to occur in plant AA synthesis based on radiolabeled glucose experiments (Loewus, 1963; Wheeler et al., 1998). Ascorbic acid biosynthesis can also occur through D-galacturonic acid, but this pathway is believed to mainly serve as a salvage mechanism during the breakdown of pectin constituents: homogalacturonan and rhamnogalacturonan I. Lorence et al. (2004) demonstrated that *myo*-inositol is also a precursor in an

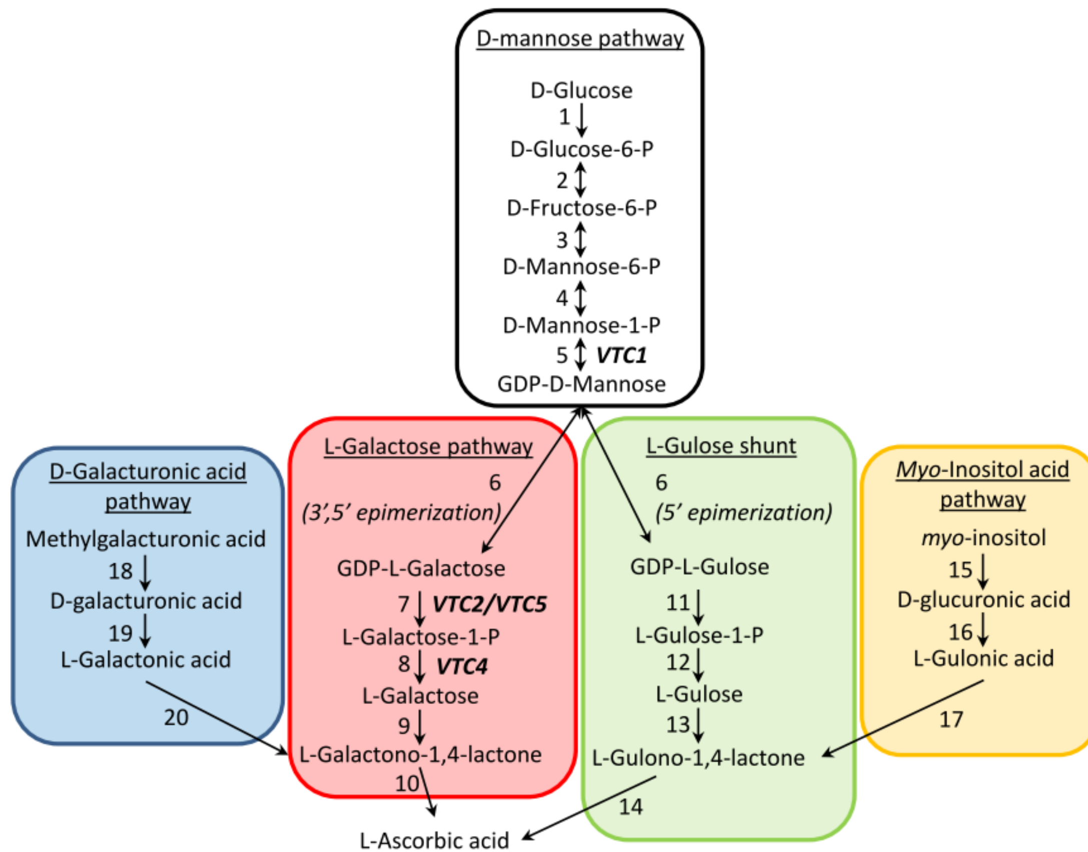


Figure 1.1 Schematic of the known pathways contributing to AA biosynthesis in plants.

After the production of D-mannose, catalyzed by hexokinase (1), phosphoglucose isomerase (2), phosphomannose isomerase (3), phosphomannose mutase (4), and GDP-D-mannose pyrophosphorylase (5) (VTC1) the product GDP-D-mannose can be funneled into the L-galactose pathway (red shaded rectangle) or the L-gulose shunt (green shaded rectangle), depending on the epimerization product of GDP-D-mannose-3,5-epimerase (6). For the L-galactose pathway, 3',5' epimerization occurs followed by catalysis involving GDP-L-galactose pyrophosphatase (7) (VTC2/VTC5), L-galactose-1-phosphate phosphatase (8) (VTC4), L-galactose dehydrogenase (9), and L-galactono-1,4-lactone dehydrogenase (10), which produces L-ascorbic acid. If 5' epimerization occurs at the GDP-D-mannose-3,5-epimerase (6) step, GDP-L-gulose is catalyzed by GDP-L-gulose pyrophosphatase (11), L-gulose-1-phosphate phosphatase (12), L-gulose dehydrogenase (13), and L-gulono-1,4-lactone dehydrogenase (14). *Myo*-inositol can contribute to plant AA (yellow shaded rectangle) through *myo*-inositol oxidase (15), glucuronic acid reductase (16), and aldonolactonase (17), which produces L-gulono-1,4,-lactone. D-galacturonic acid (salvage pathway, blue shaded box), is believed to occur through the activity of pectin methylesterase (18), galacturonic acid reductase (19), and aldonolactose producing L-galactono-1,4-lactone (20). Adapted from Hancock et al. (2005).

alternative path to AA biosynthesis. This pathway would require multiple inversions of the carbon skeleton of D-glucose. Such inversions have been previously observed, although in limited amounts. Substantial evidence for the role of the L-galactose/D-mannose pathway toward plant AA content came from the isolation of *Arabidopsis* mutants that accumulate low levels of AA (Conklin et al., 1999; Conklin et al., 2000).

1.1.3 Ascorbic acid-deficient *Arabidopsis thaliana vtc* mutants

Conklin and co-workers initially isolated four *vitamin C deficient* (*vtc*) mutants largely by virtue of their sensitivity to ozone, corresponding to four distinct loci (*VTC1*, *VTC2*, *VTC3*, and *VTC4*). The *Arabidopsis vtc1-1* mutant was shown to encode a GDP-mannose pyrophosphorylase (Conklin et al., 1996; Conklin et al., 1997). This mutant contains approximately 30% of the wild-type AA content and has sensitivity to ROS. Further characterization of the *vtc* mutants has revealed four enzymes involved in AA biosynthesis (Fig. 1.1): VTC1, a GDP-mannose pyrophosphorylase (Conklin et al., 1999); VTC2, and its homolog, VTC5, which are both GDP-L-galactose phosphorylases (Dowdle et al., 2007; Linster et al., 2007; Linster et al., 2008); VTC4, an L-galactose-1-P-phosphatase (Conklin et al., 2006); the function of VTC3 remains unknown. *vtc2-1* mutants contain approximately 30% of wild type AA and has a G to A point mutation at the predicted 3' splice site of intron 5 (Conklin et al., 2000; Jander et al., 2002). *vtc4-1* mutants contain approximately 50% of wild type AA and has a C to T point mutation changing a conserved proline residue to a leucine (Conklin et al., 2000; Conklin et al., 2006) . *vtc3-1* mutant individuals contain approximately 30% of wild type AA (Conklin et al., 2000). *vtc5-1* mutants contain a T-DNA insertion and have approximately 80% of wild type AA (Dowdle et al., 2007). Interestingly, the *vtc* mutants have varying pleiotropic phenotypes in addition to their AA-deficiencies (Table 1.1). These include altered flowering time and senescence (Barth et al., 2004; Conklin and Barth, 2004; Pavet et al., 2005; Kotchoni et al., 2009), increased resistance to pathogen attack (Pastori et al., 2003; Barth et al., 2004; Conklin and Barth, 2004; Pavet et al., 2005; Mukherjee et al., 2010), increased amounts of the phytohormones: abscisic acid (ABA; Pastori et al., 2003) and salicylic acid (SA; Mukherjee et al., 2010), and increased levels of hydrogen peroxide (Kotchoni et al., 2009; Barth et al., 2010). *vtc1-1* mutants also exhibit a stunted root phenotype when grown in the presence of ammonium (Qin et al., 2008; Kotchoni et al., 2009; Barth et al., 2010; Kempinski et al., 2011).

Table 1.1 Pleiotropic phenotypes of *Arabidopsis vtc* mutants.

Abbreviations: AmA, amino acid; ABA, abscisic acid; AA, ascorbic acid; N.D., not determined; SA, salicylic acid; WT, wild type (Kotchoni et al., 2009; Barth et al., 2004; Conklin et al., 2004; Mukherjee et al., 2010).

Genotype	Mutation WT-AmA-*vtc*	% WT AA	% WT H_2O_2	% WT SA	% WT ABA	Flowering, senescence	Resistance to virulent pathogens	Root growth in NH_4^+
vtc1-1	Pro-22-Ser	35	174	370	500	Early	Increased	Stunted
vtc2-1	Splice site	30	165	370	300	Early	Increased	Normal
vtc3-1	Unknown	30	150	250	N.D.	Early	Increased	Normal
vtc4-1	Pro-92-Leu	50	101	110	N.D.	Early	Increased	Normal
vtc5-1	Insertion	80	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

1.1.4 GDP-mannose is an important molecule for ascorbic acid biosynthesis and protein N-glycosylation

An important intermediate in AA biosynthesis is GDP-mannose, an activated sugar nucleotide. Biosynthesis of GDP-mannose requires three enzymes: phosphomannose isomerase (PMI), phosphomannose mutase (PMM), and GDP-mannose pyrophosphorylase (GMPase, VTC1; Fig. 1.1). GDP-mannose contributes to the biosynthesis of AA and different structural carbohydrates in plant cell walls, and is essential for post-translational modifications, such as protein N-glycosylation and glycosylphosphatidylinositol (GPI)-anchoring (Qian et al., 2007). GDP-mannose provides mannose, a crucial building block of the core glycan chain attached to modified proteins (Lerouge et al., 1998; Spiro, 2002).

1.1.5 Protein N-glycosylation in *Arabidopsis*

Disturbance of the protein N-glycosylation process, which takes place in the endoplasmatic reticulum (ER) lumen and in the secretory system (Silberstein and Gilmore, 1996; Helenius and Aebi, 2001), leads to a buildup of misfolded proteins in the ER and triggers the unfolded protein response (UPR). UPR is a stress response that induces the expression of genes whose products ensure proper protein folding in the ER, including calnexin, calreticulin, binding protein (BiP), and peptide disulfide isomerase (Travers et al., 2000; Martinez and Chrispeels, 2003).

Upregulation of secretion-related genes was shown to be essential for the induction of systemic acquired resistance against bacterial pathogens in Arabidopsis (Wang et al., 2005). A mutation in the *STT3a* gene encoding an essential subunit of the oligosaccharyltransferase complex causes decreased protein glycosylation, resulting in improper protein folding, binding protein (*BiP*) hyperexpression, reduced *CYCLIN B1* expression and disturbed cell cycle progression. These results suggested that the STT3a subunit is necessary for cell cycle regulation during osmotic stress (Koiwa et al., 2003). The Arabidopsis BAX inhibitor-1 (BI1) plays a pivotal role as a highly conserved survival factor that is required to delay the onset of programmed cell death (PCD) upon ER stress signaling (Watanabe and Lam, 2008). Although there is strong evidence that genes encoding secretory and vacuolar proteins are induced by hypersaline conditions (Gong et al., 2001) and that mutations in components of the secretory machinery cause osmotic sensitivity and are important for ion homeostasis (Zhu et al., 2002; Shi et al., 2003), recent data suggest that protein N-glycosylation functions beyond protein folding in the ER (Lerouxel et al., 2005; Kang et al., 2008).

It was reported previously that GMPase, encoded by *VTC1*, is a genetic determinant of NH_4^+ sensitivity. Qin et al. demonstrated that GMPase activity was inhibited by NH_4^+ and that defective protein N-glycosylation, the unfolded protein response and cell death were

downstream effects involved in the root growth inhibition in the *hsn1 Arabidopsis* mutant (Qin et al., 2008). These results were essentially confirmed by Barth and co-authors, whose data suggested that NH_4^+ hypersensitivity in *vtc1-1* is caused by disturbed N-glycosylation and associated with auxin and ethylene homeostasis and/or nitric oxide signaling (Barth et al., 2010). Finally, Li and co-authors recently presented data, indicating an association of NH_4^+ efflux at the elongation zone with the NH_4^+-mediated inhibition of primary root elongation, whereby GMPase directly or indirectly regulates this process (Li et al., 2010). Therefore, the primary cause of the stunted root growth in *vtc1-1* as well as the underlying mechanism of the defective growth response is still unclear.

In order to better understand the cause and the mechanism of the growth defect in *vtc1-1*, we addressed the following questions: (i) Do *vtc1-1* individuals suffer from cell wall or cell membrane defects *per se* or are these defects caused by the presence of NH_4^+? (ii) Are developing root cells in *vtc1-1* mutants defective in cell cycle progression? (iii) If GDP-mannose deficiency is the primary cause of NH_4^+ sensitivity, are other mutants with deficient GDP-mannose also sensitive to NH_4^+? (iv) Since NH_4^+ is known to alter cytosolic pH, do *vtc1-1* mutants respond to extracellular pH changes in the presence or absence of NH_4^+?

1.2 MATERALS AND METHODS

1.2.1 Plant material, growth and tissue culture conditions

Arabidopsis thaliana Col-0 wild type and previously described *vtc1-1*, *vtc2-1*, *vtc4-1* mutants (all in the Col-0 background) were kindly provided by Patricia Conklin (Conklin et al., 1996; Conklin et al., 2000; Conklin, 2001). Additionally, a T-DNA insertion line (SALK_029748) was obtained for the previously described *Arabidopsis PHOSPHOMANNOSE ISOMERASE 1* (*PMI*; AT3G02570) gene (Maruta et al., 2008) from the Arabidopsis Biological Resource Center. Mutant seed of *PHOSPHOMANNOSE MUTATSE* (*PMM*, AT2G45790), *pmm-12*, were kindly provided by Frank van Breusegem (Hoeberichts et al., 2008).

For assessment of root growth, seed of the wild type and mutant lines were surface-sterilized (see below) and grown on basal full strength 1x Murashige and Skoog (MS) medium without vitamins (Cat.# MSP01, Caisson Laboratories, Inc., North Logan, UT) or full strength 1x MS without ammonium nitrate (but still containing potassium nitrate) also without vitamins (Cat.# MSP05), containing 1% Phytoblend (Cat.# PTP01, Caisson Laboratories) in omni trays (Fisher Scientific, Pittsburgh, PA) as described (Barth et al. 2010). Additional experiments were performed in 1x MS without ammonium nitrate and vitamins (but still containing potassium nitrate) and with the addition of KCl, NaCl, $CaCl_2$, LiCl, mannitol, $CdCl_2$ or MES at concentrations indicated in the figures and supplemental information. Note that sucrose was omitted from all

growth media. Unless noted otherwise, the pH of the growth media was adjusted with HCl or KOH to 5.7 after making the indicated salt or buffer additions. 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} (fluorescent bulbs) 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, rosette leaves were harvested for the AA assay (see below). For AA measurements of plants grown on 1x MS, whole rosettes of two-week-old plants were collected.

1.2.2 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 (Weigel and Glazebrook, 2002).

1.2.3 Generation of *vtc1-1 VTC1* heterozygous mutants

To generate heterozygous *vtc1-1 VTC1* mutants, homozygous *vtc1-1* mutant plants were crossed with Col-0 wild-type plants. The resulting F_1 generation is heterozygous at the *VTC1* locus.

1.2.4 RNA isolation, cDNA synthesis and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from root tissue collected and pooled from seven- or 14-day-old seedlings using the Tri-Reagent protocol (Molecular Research Center, Inc., Cincinnati, OH). One microliter of total RNA was treated with 1 µL of DNaseI (Invitrogen, Carlsbad, CA) and subsequently used for reverse transcription using 10 pg of oligo(dT) primers using the first-strand cDNA synthesis kit (Invitrogen). Quantitative RT-PCR reactions were set up using 2.5 pmole of gene-specific primers and 1:10 dilutions of cDNA:DNase/RNase free water with the iQ SYBER Real-Time Master Mix (Bio-Rad, Hercules, CA) in a total volume of 10 µL. Negative controls contained water instead of reverse transciptase. In addition, reactions without template were set up to ensure that the master mix was not contaminated. Reactions were run in a Bio-Rad iCycler for 40 cycles. The threshold cycles (C_T) were calculated using iQ software

(Bio-Rad) and relative transcripts (RT) were calculated using the formula: $RT = 1/2^{CT}$. The RT values of the genes assessed were normalized to *TUBULIN2* and mean values of biological replicates were calculated. Experiments were performed at least three times. PCR fragments were separated on a 1% agarose gel stained with ethidium bromide to check for correct fragment amplification. Gene-specific primer sequences are listed in Table 1.2.

1.2.5 DAPI staining

Primary roots of seven-day-old seedlings were collected and immediately washed two times in phosphate buffered saline (PBS) buffer pH 7.4. Roots were then stained with 2.5 µg/mL DAPI (4',6-diamidino-2-phenylindole) for 20 min, washed two times again in PBS buffer, and mounted in 50% glycerol. DAPI staining was visualized with a standard UV fluorescence filter set and epifluorescence optics on a Nikon E800 microscope equipped with a CoolSNAP cf CCD camera (Photometrics, Tucson, AZ).

1.2.6 Calcofluor white aniline blue staining

Primary roots of seven-day-old seedlings were collected and immersed in 0.01% calcofluor white (Sigma-Aldrich Corp., St. Louis, MO) or 0.01% aniline blue (Sigma-Aldrich), respectively, and stained for 10 min. Seedlings were washed two times in de-ionized water and mounted in 50% glycerol. Microscopy was performed as described above.

1.2.7 Ascorbic acid assay

Leaf AA content was quantified using the iron reduction assay as described (Dowdle et al., 2007; Mukherjee et al., 2010).

1.2.8 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.

1.3 RESULTS

1.3.1 Transcript levels of ER stress genes are higher in *vtc1-1* in the presence of NH_4^+

Since GDP-mannose is involved in protein N-glycosylation, we would expect that GDP-mannose deficiency is expected to result in an upregulation of ER stress genes, enhanced PCD, and defective cell cycle proliferation in *vtc1-1* triggered by NH_4^+.

When grown in the presence of NH_4^+, *BIP* mRNA levels were more than twice as high in the *vtc1-1* mutant as in the wild type (Fig. 1.2). BIP is an ER-localized chaperone, belonging to

Table 1.2 Sequences of oligonucleotide primers used for qRT-PCR.

Primer Name	5` 3`	ATG Number
BI1_CDS-F	CAGAAGCTGGAGCTATGATTC	AT5G47120
BI1_CDS-R	CATAGTCCATGTCACCGAGGT	
BIP_CDS-F	ATCGAGGTCACATTTGAAGTGGA	AT5G42020
BIP_CDS-R	TAGAGCTCATCGTGAGACTCATCT	
CYCLINB1_RT-PCR_CK-F	AGGCTGCTTGTGGTTTAGAGAA	AT4G37490
CYCLINB1_RT-PCR_CK-R	TTGGCCGACATGAGAAGAGC	
HISTONEH4_CDS-F	ATGTCTGGTCGTGGAAAGGGAG	AT5G59970
HISTONEH4_CDS-R	ACCAAATTGCGTGTTTCCATTG	
VTC1-F	TCGCTTGAGACCATTGACT	AT2G39770
VTC1-R	TCGCTAGAGCCAGAGGAC	
TUB2-RT-F2	CTCAAGAGGTTCTCAGCAGTA	AT5G62690
TUB2-RT-R2	TCACCTTCTTCATCCGCAGTT	

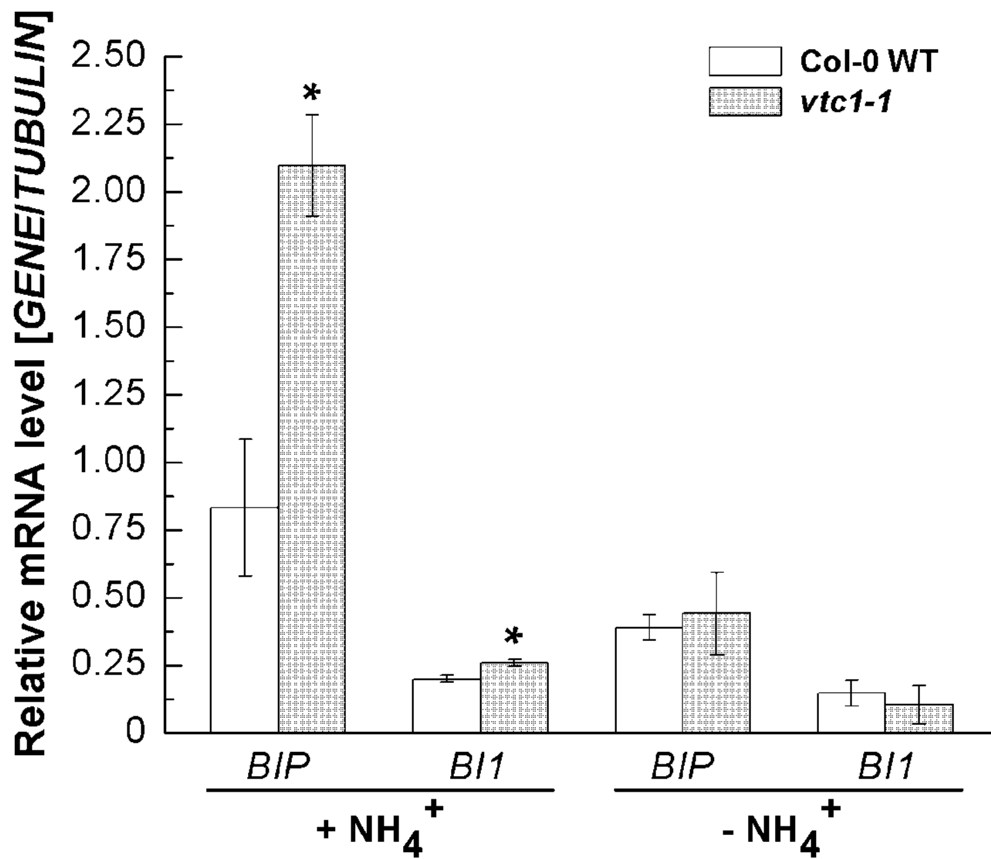


Figure 1.2 Transcript levels of the ER stress genes *BIP* (*binding protein*) and *BI1* (*BAX inhibitor-1*) in the presence and absence of NH_4^+.

Relative mRNA levels were determined in root tissue from seven-day-old Col-0 wild-type and *vtc1-1* seedlings and based on *TUBULIN*. Data indicate means ± SE of three biological replicates of each genotype and treatment. Asterisks indicate significant differences between the mutant and the wild type. * *P* < 0.05, Student's *t*-test.

the HSP70 family, which is necessary for the transport and secretion of proteins in the ER. It has been used as a common marker for UPR activation in eukaryotes, including plants (Koizumi et al., 2001; Martinez and Chrispeels, 2003). Transcript levels of *BI1* were also upregulated in *vtc1-1* compared to the wild type, although only slightly. BI1 serves as a crucial cell survival factor that is important to delay the onset of PCD upon ER stress signaling (Watanabe and Lam, 2008). In contrast, mRNA levels of *BIP* and *BI1* were the same in the wild type and *vtc1-1* in the absence of NH_4^+ (Fig. 1.2). This data suggests that the presence of NH_4^+ activates ER stress and UPR to a greater extent in *vtc1-1* than in the wild type.

1.3.2 *vtc1-1* exhibits PCD and some cell cycle defects in the presence of NH_4^+

To further examine whether enhanced root cell death in the presence of NH_4^+ occurs through necrosis or PCD, we visualized nuclei by staining wild type and *vtc1-1* roots with DAPI when plants were grown in the presence or absence of NH_4^+. PCD is characterized by morphological changes in the nucleus that are absent during necrosis. As shown in Fig. 1.3a, root nuclei in *vtc1-1* plants grown in the presence of NH_4^+ exhibited brighter fluorescence due to chromatin condensation (arrow heads in Fig. 1.3a). In contrast, nuclei of wild-type root cells and of both genotypes grown in the absence of NH_4^+ (Fig. 1.3b) appeared more round in shape and had a uniform granular appearance throughout the root. Overall cell density was strongly diminished in *vtc1-1* and abnormal nuclei were found throughout the elongation and differentiation zone (root hair zone) when the mutant was grown in the presence of NH_4^+, while the meristematic zone appeared normal. The elongation zone of the primary root was much shorter in *vtc1-1*, as root hairs were visualized within a short distance from the root tip (arrows in Fig. 1.3a).

To test whether cell cycle progression is affected in *vtc1-1*, we examined mRNA levels of the cell cycle genes *HISTONE H4* and *CYCLIN B1*. The transcript level of *HISTONE H4*, which is specifically expressed during S-phase and into G2-phase of the cell cycle (Culligan et al., 2004), was almost double the amount in *vtc1-1* compared to the wild type in the presence of NH_4^+, whereas no significant differences were found between the two genotypes in the absence of NH_4^+ (Fig. 1.3c, d). In contrast, the wild type and *vtc1-1* did not differ in the amount of *CYCLIN B1* mRNA, which is expressed during G2-phase of the cell cycle, in either treatment.

Taken together, these data suggest that NH_4^+ stress in combination with defective GMPase induces hallmarks of PCD and S-phase arrest.

1.3.3 *vtc1-1* exhibits cell wall defects and accumulates callose in the presence of NH_4^+

To determine whether the N-glycosylation defect in *vtc1-1* causes weak cell walls or whether a cell wall defect is triggered by NH_4^+, we investigated cell wall integrity by staining

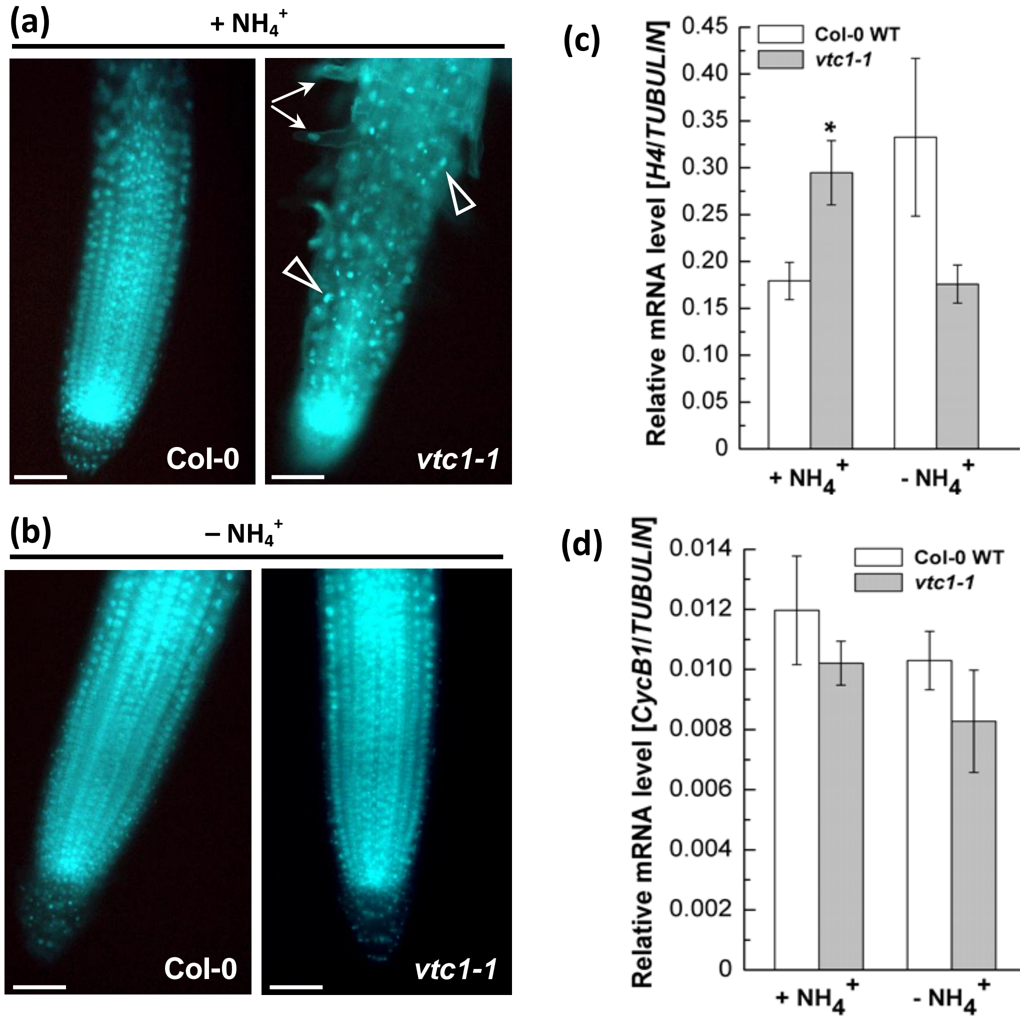


Figure 1.3 Microscopic analysis of Col-0 wild-type and *vtc1-1* roots in (a) the presence and (b) absence of NH_4^+ and (c) mRNA levels of *H4* (*HISTONE H4*) and (d) *CycB1* (*CYCLIN B1*) in the presence and absence of NH_4^+.

(a) and (b) seven-day-old roots stained with 2.5 µg/mL DAPI. Bar = 50 µm. (c) and (d) relative transcript levels based on *TUBULIN* in root tissue from seven-day-old wild-type and *vtc1-1* seedlings. Data represent means ± SE of 8 to 11 biological replicates of each genotype and treatment. Asterisks indicate significant differences between the mutant and the wild type. * $P < 0.05$, Student's *t*-test.

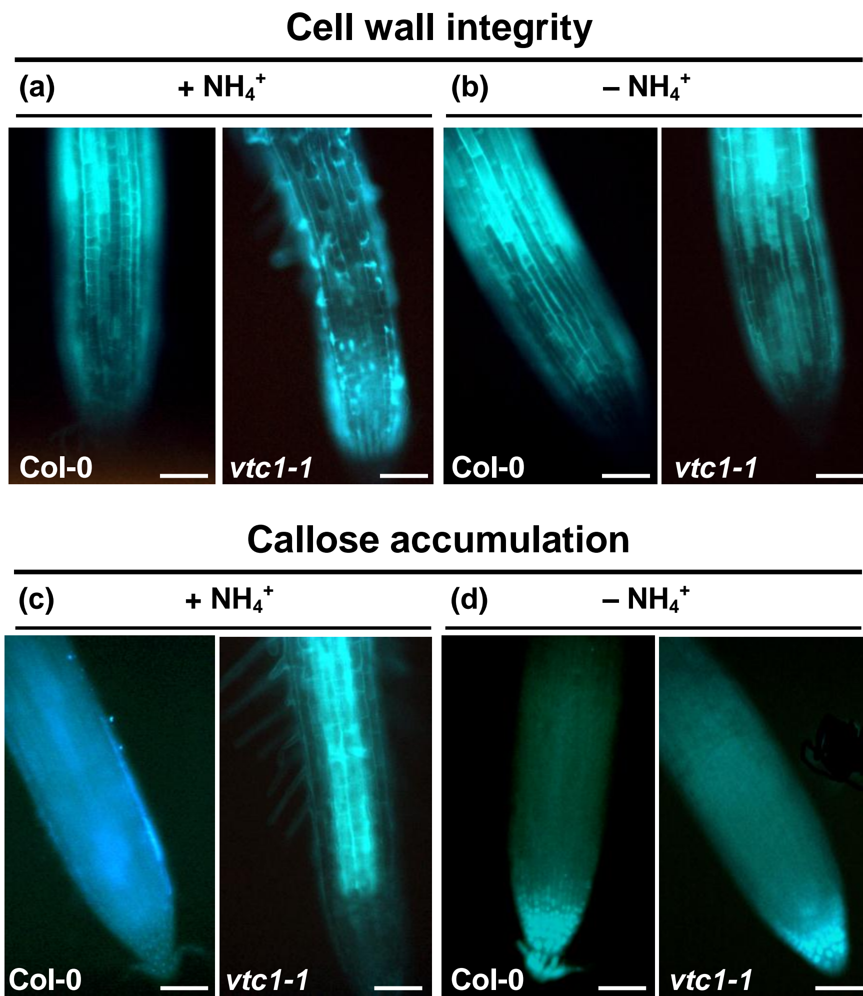


Figure 1.4 Cellulose (a, b) and callose (c, d) staining in roots of seven-day-old Col-0 wild-type and *vtc1-1* plants grown in the presence and absence of NH_4^+.

Seven-day-old roots were stained with 0.01% calcofluor white and aniline blue, respectively. Bar = 50 µm

cellulose with calcofluor white and tested for callose deposition, a marker of cell wall and/or membrane defects, using aniline blue. Fig. 1.4a shows strongly reduced calcofluor white fluorescence in *vtc1-1* grown in the presence of NH_4^+, whereas homogenous staining was observed in the wild type and in primary roots of both genotypes in the absence of NH_4^+ (Fig. 1.4b). Defective cell walls correlated with callose deposition particularly in the central cylinder of *vtc1-1* in the presence of NH_4^+, while aniline blue staining was more diffuse in the wild type (Fig. 1.4c) and in both genotypes grown in the absence of NH_4^+ (Fig. 1.4d). Collectively, the data suggest that cell wall and membrane defects are triggered by NH_4^+ stress in *vtc1-1* rather than the mutant has weakened cell walls or membranes sensitive to NH_4^+.

1.3.4 *pmi* and *pmm* mutants are not NH_4^+-sensitive

If NH_4^+ sensitivity in *vtc1-1* is caused by GDP-mannose deficiency, we would expect other mutants contributing to GDP-mannose biosynthesis to also show a stunted root phenotype in the presence of NH_4^+. The *pmi-1* and *pmm-12* mutants have defects in genes directly upstream of *VTC1* in the D-mannose/L-galactose pathway. Genetic defects in *VTC1*, *PMI* and *PMM* have been shown to result in decreased activity of the respective enzymes, indirectly demonstrating a reduced accumulation of GDP-mannose (Fig. 1.1; Conklin et al., 1999; Hoeberichts et al., 2008; Maruta et al., 2008). When grown on soil, the *pmi-1* and *pmm-12* mutants contain approximately 80% of the wild-type AA content (Fig. 1.5). When grown in the presence of NH_4^+, primary roots of *pmi-1* and *pmm-12* were similar or slightly longer than in the wild type, respectively, whereas root growth was strongly inhibited in *vtc1-1*, as expected (Fig. 1.6a). In the absence of NH_4^+, primary roots were slightly longer in *pmi-1* and *pmm-12* mutants compared to the wild type and *vtc1-1* (Fig. 1.6b). The *pmm-12* mutant is temperature-sensitive, although total PMM activity and protein levels were considerably reduced at the permissible temperature of 16°C (Hoeberichts et al., 2008). In the absence of NH_4^+ but at the restrictive temperature of 28°C (compared to 23°C used in our experiments, see Materials and Methods), *pmm-12* root and overall growth was strongly inhibited, as expected. However, this was not the case for *pmi-1* and *vtc1-1*, which developed longer than wild-type primary roots or roots similar to the wild type, respectively (Fig. 1.6c). Finally, to test whether the defect in GDP-mannose/AA biosynthesis in these three mutants causes heightened susceptibility to oxidative stress, we grew *pmi-1*, *pmm-12*, and *vtc1-1* in comparison to the wild type in the absence of NH_4^+ but in the presence of Cd (Fig. 1.6d). In comparison to the control (Fig. 1.6b), all four genotypes exhibited root growth inhibition in the presence of Cd (Fig. 1.6d). However, none of the mutants was more susceptible than the wild type. In fact, *pmm-12* developed somewhat longer roots than the other genotypes in the presence of Cd (Fig. 1.6d). In summary, these results suggest that the inferred low level of

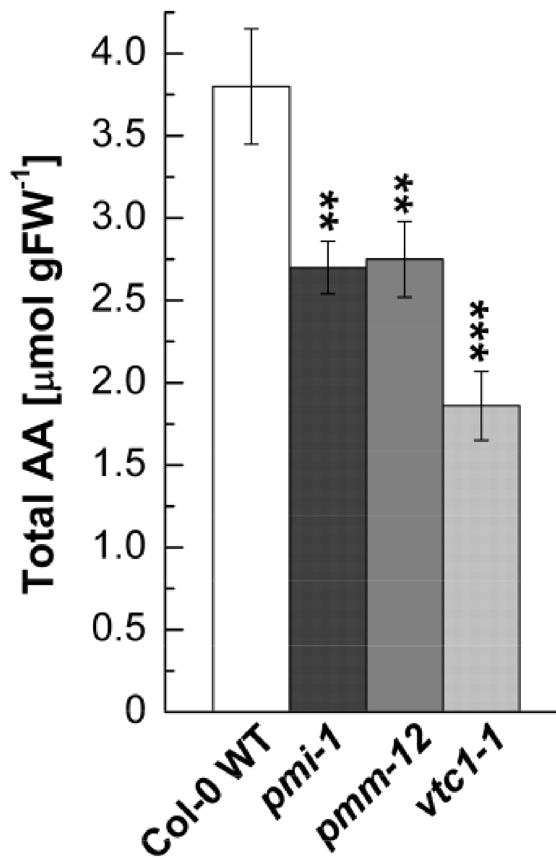


Figure 1.5 Total ascorbic acid (AA) content in Col-0 wild-type plants and *pmi-1*, *pmm-21*, and *vtc1-1* mutants grown on soil.

Ascorbic acid content was measured in whole rosettes of three-week-old plants. Means ± SE of seven individual plants are shown. Asterisks indicate significant differences between individual mutants and the wild type. ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

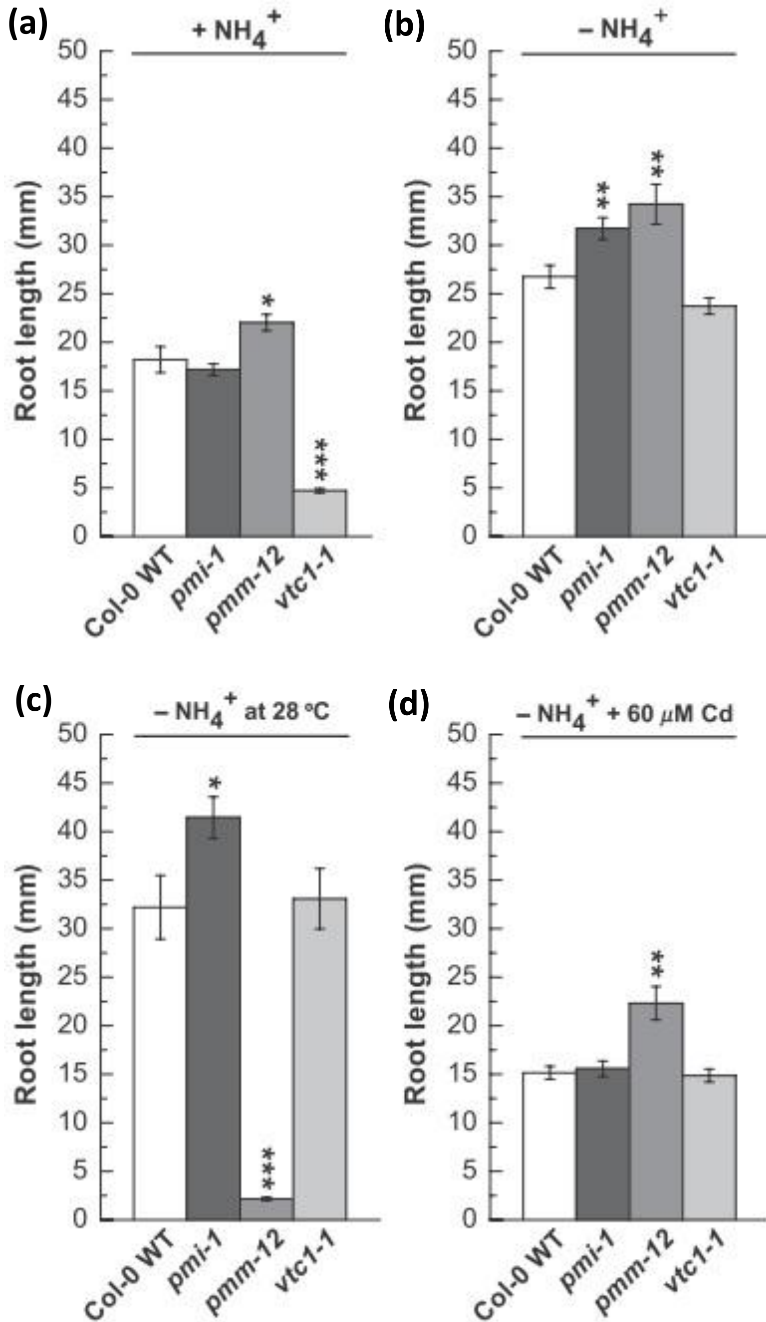


Figure 1.6 Primary root length of Col-0 wild type, *pmi-1*, *pmm-12* and *vtc1-1* mutants in the (a) presence and (b) absence of NH_4^+, (c) during heat stress, and (d) in the presence of Cd.
Primary root length was measured when seedlings were seven days old. Results illustrate means ± SE of 12 to 24 individual seedlings per genotype and treatment. Asterisks indicate significant differences between individual mutants and the wild type. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

GDP-mannose does not cause general sensitivity to NH_4^+ and that GDP-mannose deficiency and the resulting downstream effects (ER stress, UPR, cell wall, cell membrane defects, see above) are not the primary cause of the root growth inhibition in *vtc1-1* in the presence of NH_4^+. Since the *pmi-1* and *pmm-12* mutants behave differently from *vtc1-1* when grown with NH_4^+, we hypothesize that the growth defect in *vtc1-1* is inherent to the specific mutation in the GMPase enzyme.

1.3.5 Primary root growth is recovered in *vtc1-1* at pH 7.0 in the presence of NH_4^+

NH_4^+ is known to cause alkalization of the cytosol (Britto, 2005) and, depending on the concentration and pH of the external medium, presumably alters proton and/or redox homeostasis within the cell. Therefore, we investigated whether the *vtc1-1* mutant responds to pH changes in the growth medium in the absence and presence of NH_4^+. Thus, we would expect that root growth is the same in the wild type and *vtc1-1* at various pH values in the absence of NH_4^+. However, we predict that root growth is different in *vtc1-1* from the wild type in the presence of NH_4^+.

When the wild type and *vtc1-1* mutants were grown at pH 4.0, 5.0, 5.7, 7.0, 8.0 and 9.0 in the absence of NH_4^+, root development was comparable between the two genotypes and only small changes in overall primary root length were detected (Fig. 1.7a). However, in the presence of 20.61 mM NH_4Cl (the concentration of NH_4^+ present in 1x MS), primary root growth was strongly affected in both genotypes, but even more in *vtc1-1*. Primary root length was maximal in the wild type and *vtc1-1* at pH 7.0, which represents a remarkable recovery of root development in *vtc1-1* compared to the low pH ranges. Note that we found similar results in the presence of the buffering agent MES (Fig. 1.8). However, root development is not fully complemented in the mutant compared to the wild type (Fig. 1.7b). When plants were grown on media with alkaline pH values (close to the pK_a of NH_4^+), primary root growth was strongly inhibited in both genotypes. The numbers above the pH values in Fig. 1.7b indicate the calculated concentrations of NH_3 present in the medium using the Henderson-Hasselbalch equation (Table 1.3). Note the drastic changes in free NH_3 with increasing pH. Similar calculations were performed for the growth of the wild type and *vtc1-1* when grown in increasing concentrations of NH_4Cl at pH 5.7 (Fig. 1.9). Finally, since it is known that NH_4^+ and K^+ ions compete during uptake via K^+ transporters and channels and that NH_4^+ toxicity may be alleviated by the addition of K^+ (Cao et al., 1993; ten Hoopen et al., 2010), we examined primary root development of the wild type and *vtc1-1* in the presence of NH_4^+ and KCl. We also investigated whether the addition of $CaCl_2$ would suppress the root growth defect in *vtc1-1* in the presence of NH_4^+, because Ca^{2+} has been shown to rescue salt-sensitive mutants (Liu and Zhu, 1997). As indicated in Fig. 1.10a, root development did not recover in *vtc1-1* when grown

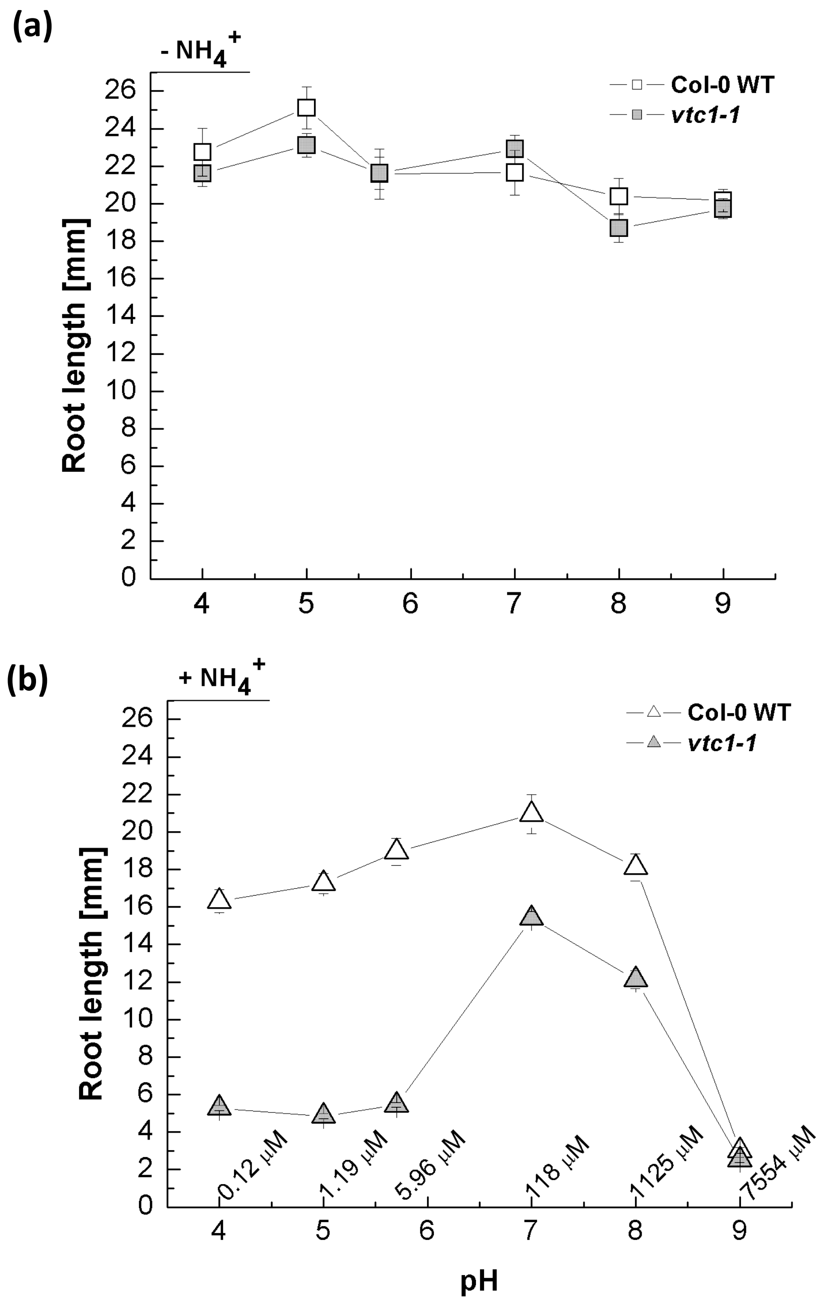


Figure 1.7 Primary root lengths of seven-day-old Col-0 wild-type and *vtc1-1* seedlings in the (a) absence and (b) presence of NH_4^+ in growth media ranging from acidic to alkaline pH values.
Data represent means ± SE of 42 to 59 individual seedlings per genotype and treatment. Numbers along abscissa in (b) represent calculated NH_3 concentrations at corresponding pH values (Table 1.3).

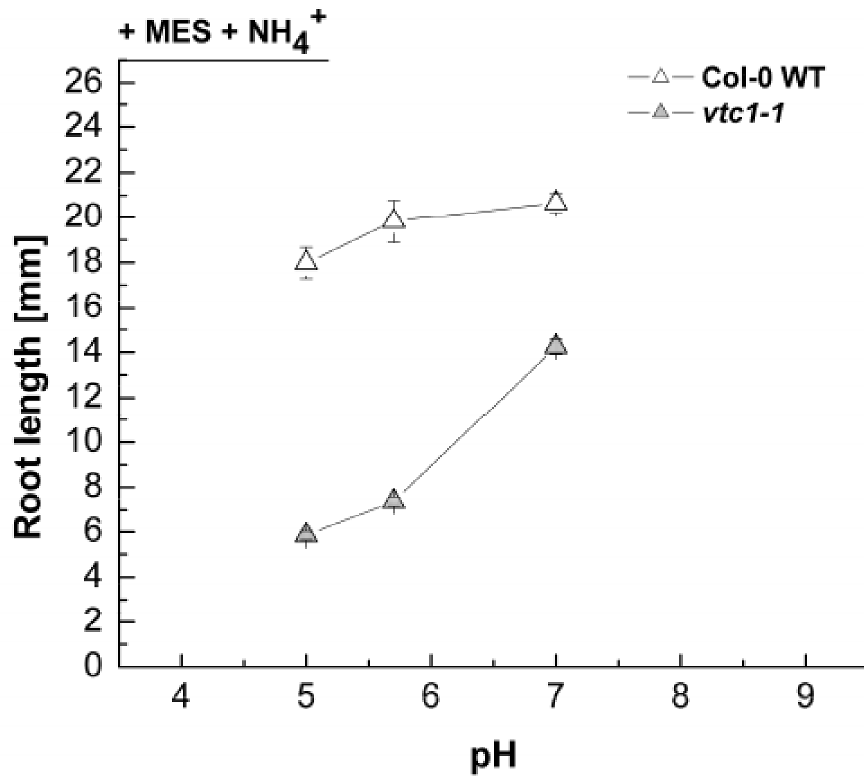


Figure 1.8 Primary root length of seven-day-old Col-0 wild-type and *vtc1-1* seedlings in the presence of NH_4^+ and the buffering agent MES (2.5 mM) in tissue culture media at pH 5.0, 5.7 and 7.0.
Data represent means ± SE of 40 to 50 individual seedlings per genotype and treatment.

Table 1.3 Calculated molar concentrations of NH_3 and NH_4^+ in 1x MS media from pH 1 to pH 11 using the Henderson-Hasselbalch equation

(pH = pKa + log($[NH_3]/[NH_4^+]$)). The pKa of NH_4^+ is 9.24.

pH	pH-pKa	$(NH_3)/(NH_4^+)$	$[H^+]$	$[NH_3]$	$[NH_4]$
1	-8.24	5.7544E-09	0.1	1.19E-10	0.02061
2	-7.24	5.7544E-08	0.01	1.19E-09	0.02061
3	-6.24	5.7544E-07	0.001	1.19E-08	0.02061
4	-5.24	5.7544E-06	0.0001	1.19E-07	0.02061
5	-4.24	5.7544E-05	0.00001	1.19E-06	0.020609
5.7	-3.54	0.000288403	1.99526E-06	5.962E-06	0.020604
6	-3.24	0.00057544	0.000001	1.189E-05	0.020598
6.05	-3.19	0.000645654	8.91251E-07	1.334E-05	0.020597
6.1	-3.14	0.000724436	7.94328E-07	1.497E-05	0.020595
6.15	-3.09	0.000812831	7.07946E-07	1.68E-05	0.020593
6.2	-3.04	0.000912011	6.30957E-07	1.884E-05	0.020591
6.25	-2.99	0.001023293	5.62341E-07	2.114E-05	0.020589
6.3	-2.94	0.001148154	5.01187E-07	2.372E-05	0.020586
6.35	-2.89	0.00128825	4.46684E-07	2.661E-05	0.020583
6.4	-2.84	0.00144544	3.98107E-07	2.985E-05	0.02058
6.45	-2.79	0.00162181	3.54813E-07	3.348E-05	0.020577
6.5	-2.74	0.001819701	3.16228E-07	3.756E-05	0.020572
6.55	-2.69	0.002041738	2.81838E-07	4.214E-05	0.020568
6.6	-2.64	0.002290868	2.51189E-07	4.727E-05	0.020563
6.65	-2.59	0.002570396	2.23872E-07	5.302E-05	0.020557
6.7	-2.54	0.002884032	1.99526E-07	5.947E-05	0.020551
6.75	-2.49	0.003235937	1.77828E-07	6.67E-05	0.020543
6.8	-2.44	0.003630781	1.58489E-07	7.481E-05	0.020535
6.85	-2.39	0.004073803	1.41254E-07	8.39E-05	0.020526
6.9	-2.34	0.004570882	1.25893E-07	9.409E-05	0.020516
6.95	-2.29	0.005128614	1.12202E-07	0.0001055	0.020504
7	-2.24	0.005754399	0.0000001	0.0001183	0.020492
7.05	-2.19	0.006456542	8.91251E-08	0.0001327	0.020477
7.1	-2.14	0.00724436	7.94328E-08	0.0001487	0.020461
7.15	-2.09	0.008128305	7.07946E-08	0.0001667	0.020443
7.2	-2.04	0.009120108	6.30957E-08	0.0001869	0.020423
7.25	-1.99	0.01023293	5.62341E-08	0.0002095	0.020401
7.3	-1.94	0.011481536	5.01187E-08	0.0002347	0.020375
7.35	-1.89	0.012882496	4.46684E-08	0.000263	0.020347
7.4	-1.84	0.014454398	3.98107E-08	0.0002947	0.020315
7.45	-1.79	0.016218101	3.54813E-08	0.00033	0.02028

7.5	-1.74	0.018197009	3.16228E-08	0.0003696	0.02024
7.55	-1.69	0.020417379	2.81838E-08	0.0004138	0.020196
7.6	-1.64	0.022908677	2.51189E-08	0.0004631	0.020147
7.65	-1.59	0.025703958	2.23872E-08	0.0005182	0.020092
7.7	-1.54	0.028840315	1.99526E-08	0.0005797	0.02003
7.75	-1.49	0.032359366	1.77828E-08	0.0006482	0.019962
7.8	-1.44	0.036307805	1.58489E-08	0.0007245	0.019885
7.85	-1.39	0.040738028	1.41254E-08	0.0008095	0.019801
7.9	-1.34	0.045708819	1.25893E-08	0.0009039	0.019706
7.95	-1.29	0.051286138	1.12202E-08	0.0010089	0.019601
8	-1.24	0.057543994	1E-08	0.0011253	0.019485
8.05	-1.19	0.064565423	8.91251E-09	0.0012542	0.019356
8.1	-1.14	0.072443596	7.94328E-09	0.0013969	0.019213
8.15	-1.09	0.081283052	7.07946E-09	0.0015546	0.019055
8.2	-1.04	0.091201084	6.30957E-09	0.0017284	0.018882
8.25	-0.99	0.102329299	5.62341E-09	0.0019197	0.01869
8.3	-0.94	0.114815362	5.01187E-09	0.0021298	0.01848
8.35	-0.89	0.128824955	4.46684E-09	0.0023601	0.01825
8.4	-0.84	0.144543977	3.98107E-09	0.0026117	0.017998
8.45	-0.79	0.16218101	3.54813E-09	0.0028859	0.017724
8.5	-0.74	0.181970086	3.16228E-09	0.0031838	0.017426
8.55	-0.69	0.204173794	2.81838E-09	0.0035064	0.017104
8.6	-0.64	0.229086765	2.51189E-09	0.0038545	0.016755
8.65	-0.59	0.257039578	2.23872E-09	0.0042287	0.016381
8.7	-0.54	0.28840315	1.99526E-09	0.0046292	0.015981
8.75	-0.49	0.323593657	1.77828E-09	0.0050559	0.015554
8.8	-0.44	0.363078055	1.58489E-09	0.0055085	0.015101
8.85	-0.39	0.407380278	1.41254E-09	0.0059861	0.014624
8.9	-0.34	0.45708819	1.25893E-09	0.0064874	0.014123
8.95	-0.29	0.512861384	1.12202E-09	0.0070106	0.013599
9	-0.24	0.575439937	1E-09	0.0075536	0.013056
9.05	-0.19	0.645654229	8.91251E-10	0.0081137	0.012496
9.1	-0.14	0.72443596	7.94328E-10	0.0086879	0.011922
9.15	-0.09	0.812830516	7.07946E-10	0.0092726	0.011337
9.2	-0.04	0.912010839	6.30957E-10	0.0098644	0.010746
9.25	0.01	1.023292992	5.62341E-10	0.0104593	0.010151
9.3	0.06	1.148153621	5.01187E-10	0.0110534	0.009557
9.35	0.11	1.288249552	4.46684E-10	0.0116429	0.008967
9.4	0.16	1.445439771	3.98107E-10	0.0122238	0.008386
9.45	0.21	1.621810097	3.54813E-10	0.0127928	0.007817

9.5	0.26	1.819700859	3.16228E-10	0.0133464	0.007264
9.55	0.31	2.041737945	2.81838E-10	0.0138818	0.006728
9.6	0.36	2.290867653	2.51189E-10	0.0143965	0.006213
9.65	0.41	2.570395783	2.23872E-10	0.0148885	0.005721
9.7	0.46	2.884031503	1.99526E-10	0.0153563	0.005254
9.75	0.51	3.235936569	1.77828E-10	0.0157987	0.004811
9.8	0.56	3.630780548	1.58489E-10	0.016215	0.004395
9.85	0.61	4.073802778	1.41254E-10	0.016605	0.004005
9.9	0.66	4.570881896	1.25893E-10	0.0169687	0.003641
9.95	0.71	5.12861384	1.12202E-10	0.0173065	0.003303
10	0.76	5.754399373	1E-10	0.0176192	0.002991
10.05	0.81	6.45654229	8.91251E-11	0.0179075	0.002702
10.1	0.86	7.244359601	7.94328E-11	0.0181726	0.002437
10.15	0.91	8.128305162	7.07946E-11	0.0184155	0.002194
10.2	0.96	9.120108394	6.30957E-11	0.0186376	0.001972
10.25	1.01	10.23292992	5.62341E-11	0.01884	0.00177
10.3	1.06	11.48153621	5.01187E-11	0.0190242	0.001586
10.35	1.11	12.88249552	4.46684E-11	0.0191914	0.001419
10.4	1.16	14.45439771	3.98107E-11	0.019343	0.001267
10.45	1.21	16.21810097	3.54813E-11	0.0194801	0.00113
10.5	1.26	18.19700859	3.16228E-11	0.0196039	0.001006
10.55	1.31	20.41737945	2.81838E-11	0.0197156	0.000894
10.6	1.36	22.90867653	2.51189E-11	0.0198162	0.000794
10.65	1.41	25.70395783	2.23872E-11	0.0199067	0.000703
10.7	1.46	28.84031503	1.99526E-11	0.0199882	0.000622
10.75	1.51	32.35936569	1.77828E-11	0.0200613	0.000549
10.8	1.56	36.30780548	1.58489E-11	0.0201269	0.000483
10.85	1.61	40.73802778	1.41254E-11	0.0201857	0.000424
10.9	1.66	45.70881896	1.25893E-11	0.0202385	0.000372
10.95	1.71	51.2861384	1.12202E-11	0.0202857	0.000324
11	1.76	57.54399373	1E-11	0.020328	0.000282

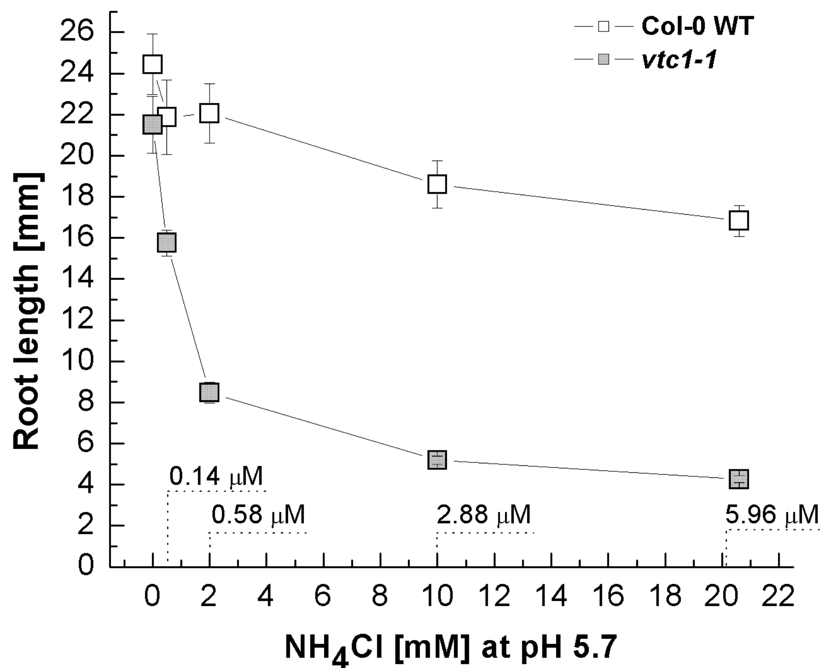
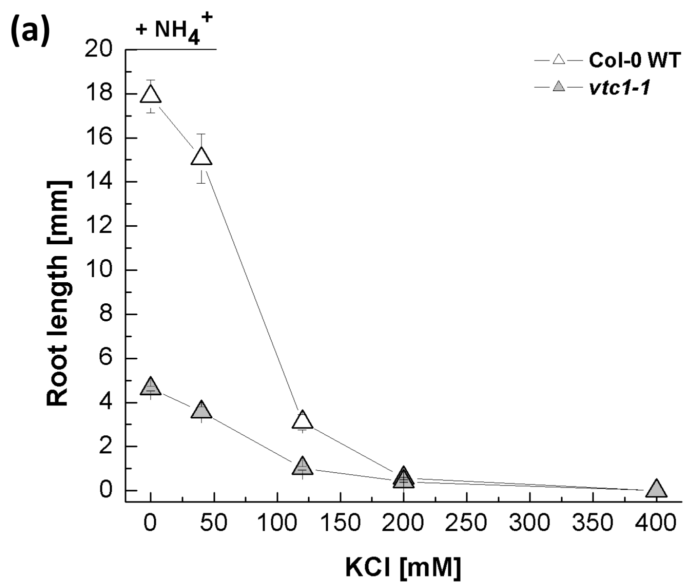


Figure 1.9 Primary root length of seven-day-old Col-0 wild type and *vtc1-1* in the presence of increasing concentrations of NH_4Cl.

Data represent means ± SE of 15 to 20 individual seedlings per genotype and concentration. Numbers on abscissa indicate calculated NH_3 concentrations at corresponding NH_4Cl concentrations.

(a)



(b)

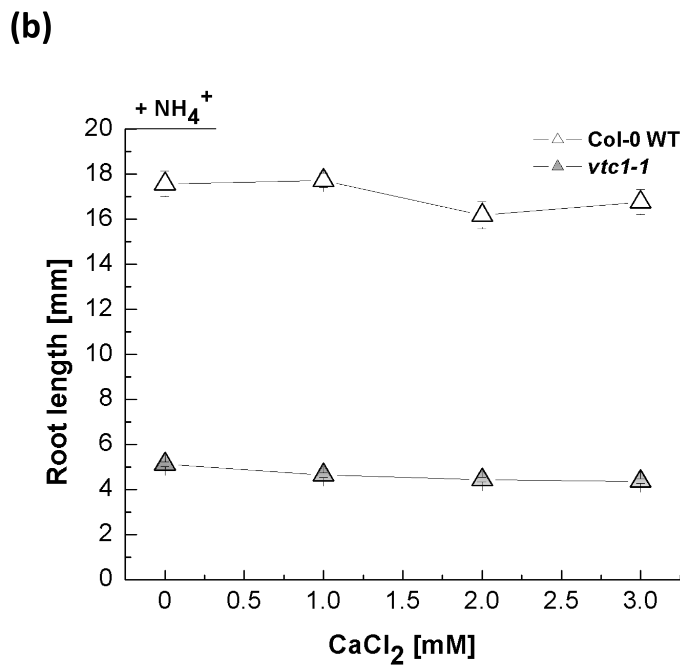


Figure 1.10 Primary root length of Col-0 wild-type plants and *vtc1-1* mutants in the presence of NH_4^+ and (a) KCl or (b) $CaCl_2$.

Data illustrate means ± SE of 49 to 60 seven-day-old individual seedlings.

in the presence of NH_4^+ and KCl. Likewise, *vtc1-1* exhibited stunted root growth compared to the wild type in the presence of both NH_4^+ and $CaCl_2$ (Fig. 1.10b). We also tested the response of *vtc1-1*, GDP-mannose-deficient and AA-deficient mutants compared to the wild type to salt and osmotic stress (Fig. 1.11). While *vtc1-1*, *vtc2-1* and *vtc4-1* were somewhat more susceptible to NaCl (Fig. 1.11a) and mannitol (Fig. 1.11b) than the wild type, this was not the case for *pmm-12* and *pmm-21*. None of the mutants were hypersensitive to KCl (Fig. 1.11c) or LiCl (Fig. 1.11d).

In conclusion, our data demonstrate that the short-root phenotype developed by *vtc1-1* can be recovered to almost wild-type levels in the presence of NH_4^+ when the pH of the growth medium is neutral. However, root growth cannot be restored by the addition of K^+ or Ca^{2+} cations. This suggests that in *vtc1-1*, NH_4^+ toxicity is K^+- and Ca^{2+}-independent but pH-dependent, presumably affecting NH_4^+ and cellular properties (e.g. cell division and differentiation, PCD, metabolic enzymes; Foyer and Noctor, [2011]), and thus influencing growth and developmental properties.

1.3.6 Root development in the presence of NH_4^+ is fully recovered in *vtc1-1* containing one wild-type copy of *VTC1*

Our results presented in Fig. 1.8 suggest that the point mutation in GMPase in *vtc1-1* makes the enzyme more susceptible to intracellular pH changes evoked by the presence of NH_4^+. In order to obtain first insights into the molecular properties of GMPase at the restrictive pH of 5.7, we examined a possible correlation between root development and *VTC1* mRNA levels when *vtc1-1* contains a wild-type copy of the *VTC1* gene and is grown at pH of 5.7 in the presence of NH_4^+.

As is shown in Fig. 1.12a, *vtc1-1 VTC1* heterozygous mutants form primary roots similar to the wild type, while *vtc1-1* homozygous mutants have strongly reduced root growth in the presence of NH_4^+, as expected. *VTC1* mRNA was significantly lower by 40% in *vtc1-1* homozygous mutants and was reduced by 70% in *vtc1-1 VTC1* heterozygotes compared to the wild type (Fig. 1.12b). No significant differences in *VTC1* transcript levels were observed in the absence of NH_4^+ (data not shown). Note that AA levels in rosettes of plants grown in the presence of NH_4^+ were similar in the wild type and *vtc1-1 VTC1* heterozygotes and were approximately 50% of the wild type in *vtc1-1* mutants (Fig. 1.13). This is in contrast to plants grown on soil (no NH_4^+ stress), where *vtc1-1 VTC1* heterozygotes have an AA content that is intermediate between the wild type and *vtc1-1* (Conklin et al., 1996).

In sum, our results suggest that the introduction of one wild-type *VTC1* copy into *vtc1-1* is sufficient to fully recover root development in *vtc1-1* at the restrictive pH in the presence of NH_4^+. Our data also suggest a regulatory mechanism at the *VTC1* mRNA level in response to NH_4^+/pH stress.

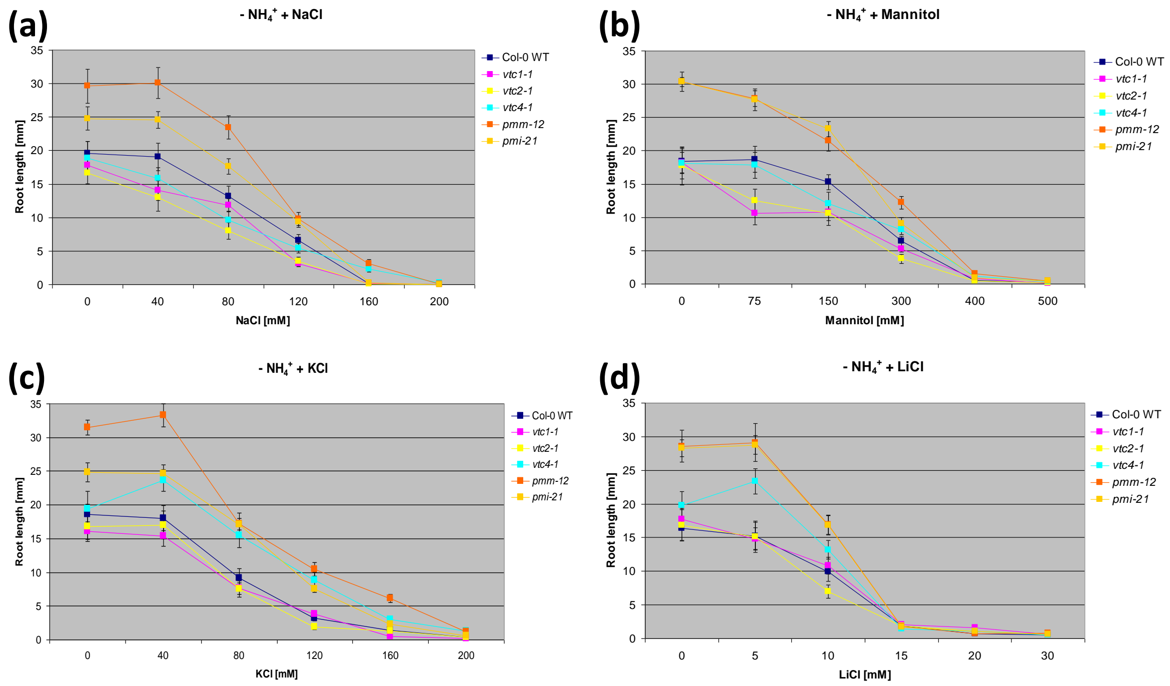
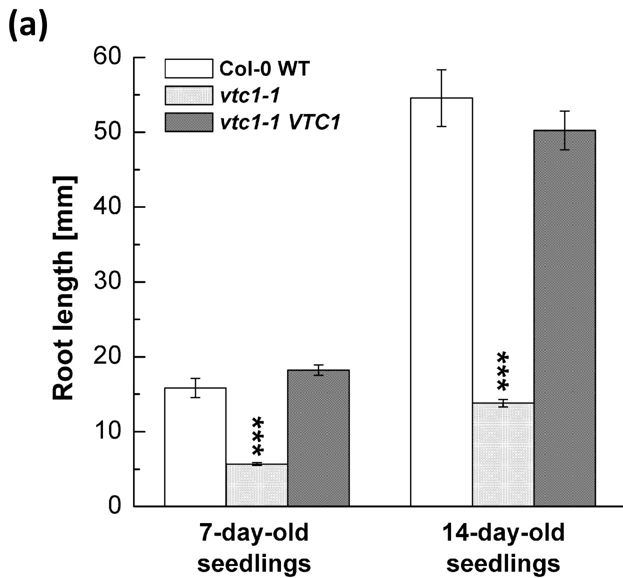


Figure 1.11 Primary root length of Col-0 wild-type plants and ascorbic acid-deficient mutants grown in the absence of NH_4^+ and in the presence of increasing concentrations of (a) NaCl, (b) mannitol, (c) KCl or (d) LiCl.

Results show means ± SE of 21 to 26 seven-day-old individual seedlings.



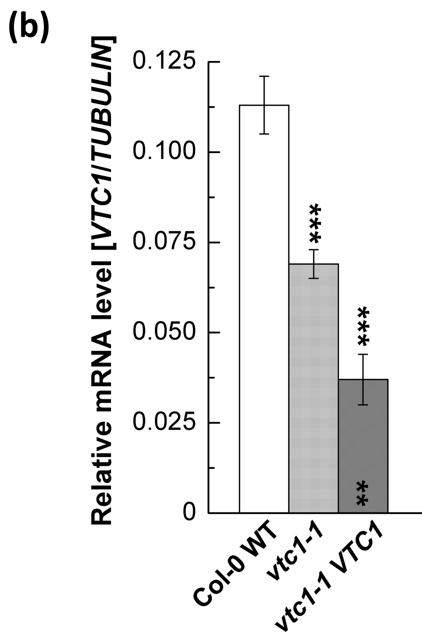


Figure 1.12 Phenotypic and molecular characterization of Col-0 wild type, *vtc1-1* homozygous and *vtc1-1 VTC1* heterozygous mutants.

(a) Primary root length of seven- and 14-day-old seedlings in the presence of NH_4^+. (b) Relative mRNA levels of *VTC1* based on *TUBULIN* in 14-day-old seedlings grown in the presence of NH_4^+. Results display means ± SE of 14 to 28 individual seedlings per genotype in (a) and of three to seven biological replicates in (b). Asterisks on top of bars indicate significant differences between mutants and the wild type. Asterisks on bottom of bar indicate significant difference between *vtc1-1* and *vtc1-1 VTC1*. ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

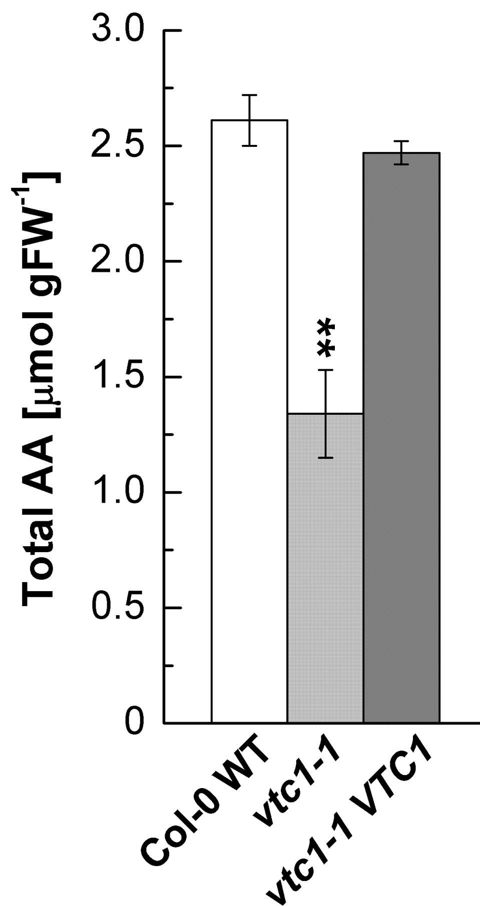


Figure 1.13 Total ascorbic acid (AA) content in Col-0 wild-type plants, *vtc1-1* homozygous and *vtc1-1 VTC1* heterozygous mutants grown in the presence of NH_4^+.

Ascorbic acid content was measured in whole rosettes of two-week-old plants. Means ± SE of four biological replicates are shown. Asterisks indicate significant differences between individual mutant and the wild type. ** $P < 0.01$, Student's *t*-test.

1.4 DISCUSSION

1.4.1 Impaired GDP-mannose biosynthesis, resulting in ER stress and defective protein N-glycosylation contributes to but is not the primary cause of NH_4^+ sensitivity in *vtc1-1*

It has been suggested previously that GMPase is a genetic determinant of NH_4^+ sensitivity in *Arabidopsis*, as has been demonstrated by the conditional hypersensitivity of two GMPase mutant alleles, *hsn1* and *vtc1-1* (Qin et al., 2008; Barth et al., 2010). Two additional GMPase mutant alleles, *cyt1-1* and *cyt2-1*, were identified, displaying severe defects in protein N-glycosylation, causing embryo lethality (Lukowitz et al., 2001). Initial investigations suggested that defective protein N-glycosylation, caused by low levels of GDP-mannose (Fig. 1.1), in the *hsn1* and *vtc1-1* mutants are linked to the NH_4^+ hypersensitivity in these mutants (Qin et al., 2008; Barth et al., 2010). Therefore, we asked whether other mutants impaired in GDP-mannose biosynthesis also exhibit NH_4^+ sensitivity. The *pmi-1* and *pmm-12* mutants act directly upstream of *vtc1-1* and contribute to GDP-mannose biosynthesis (Fig. 1.1). Surprisingly, root growth was the same in *pmi-1* and *pmm-12* mutants and the wild type in the presence of NH_4^+ (Fig. 1.6a), suggesting that GDP-mannose deficiency is not the sole cause of NH_4^+ sensitivity. It should be noted, however, that defective protein N-glycosylation in *pmm-12* causes temperature sensitivity (Hoeberichts et al., 2008), while this is not the case in *pmi-1* and *vtc1-1* mutants (Fig. 1.6c). Nevertheless, *pmm-12* mutants have decreased PMM activity and protein levels at permissible temperature (Hoeberichts et al., 2008), which is also evident in the significantly lower AA content in plants grown on soil in the absence of NH_4^+ stress (Fig. 1.5).

However, we cannot rule out that GDP-mannose deficiency, and thus defective protein N-glycosylation, contributes to the root growth defect in *vtc1-1* in the presence of NH_4^+. This is supported by the following facts: (i) ER stress genes are upregulated in *vtc1-1* (Fig. 1.2), (ii) cell cycle progression is arrested in S-phase in *vtc1-1* (Fig. 1.3c, d), and (iii) cell wall and cell membrane integrity is strongly impaired in *vtc1-1* in the presence of NH_4^+ (Fig. 1.4). The binding protein (BiP) and BAX-inhibitor 1 (BI1) are induced in response to treatment of *Arabidopsis* seedlings with tunicamycin, an inhibitor of N-linked protein glycosylation and an inducer of ER stress. Tunicamycin causes strong inhibition of root growth in wild-type seedlings (Watanabe and Lam, 2008), resembling the *vtc1-1* short-root phenotype in the presence of NH_4^+ (Barth et al., 2010). It has been previously proposed that BI1 is a critical survival factor for the suppression of PCD induced by ER stress (Watanabe and Lam, 2008). Note that BI1 is not markedly upregulated in *vtc1-1* in the presence of NH_4^+ (Fig. 1.2), suggesting that the inability of *vtc1-1* to substantially induce BI1 could promote the onset of PCD and compromise stress tolerance to NH_4^+ (Fig. 1.3a). Although the exact chain of events is currently unclear, it is possible that these apoptotic regulatory mechanisms modulate the level of cell cycle-

controlling proteins, thereby affecting DNA synthesis in *vtc1-1* in the presence of NH_4^+. This is supported by our data, which show that cell cycle progression is arrested in S-phase in *vtc1-1* under NH_4^+ stress (Fig. 1.3c, d) and by results by Li and co-workers who reported reduced meristematic cell division and/or meristem cell size in the wild type grown in the presence of NH_4^+ (Li et al., 2010). Finally, GDP-mannose functions beyond protein N-glycosylation and is important for cell wall polysaccharide biosynthesis (Fig. 1.1; Bonin et al., 1997). In fact, our data suggest that NH_4^+ induces defects in cell wall formation, while *vtc1-1* does not have weakened cell walls *per se* (Fig. 1.4a, b). Defective cell wall development in *vtc1-1* in the presence of NH_4^+ correlates with the accumulation of callose in *vtc1-1* (Fig. 1.4c), suggesting that NH_4^+ causes plasma membrane defects (see below). Callose is deposited outside the plasma membrane and may serve as a defense response to maintain structural integrity and osmotic conditions (Cresti and van Went, 1976). Since cell walls (and callose) are synthesized at the plasma membrane (Doblin et al., 2002; Saxena and Brown, 2005), altered membrane integrity in conjunction with a lack of complex N-glycans is likely to result in insufficient structural polysaccharides and glycoproteins, thereby impacting cell wall formation and thus growth in *vtc1-1* in the presence of NH_4^+. This is supported by the fact that GMPase is essential for cell wall integrity, morphogenesis and viability of *Aspergillus fumigatus* (Jiang et al., 2008). Furthermore, *Arabidopsis* mutants with defective N-glycosylation, such as *stt3a-1* and *stt3a-2* with defects in one of the subunits of an oligosaccharyltransferase complex, and *cgl1*, a mutant defective in N-glycan maturation, suffer cell-wall biosynthesis defects in response to salt stress (Koiwa et al., 2003; Kang et al., 2008). Note, however, that *cgl1* and *stt3a* single mutants do not exhibit any growth defects in the absence of stress and do not display stunted root growth in the presence of NH_4^+ (Frank et al., 2008). Interestingly, despite its N-glycosylation defect, *vtc1-1* is not hypersensitive to salt or osmotic stress (Fig. 1.11a, b). Although *vtc1-1* is somewhat susceptible to salt and osmotic stress in comparison to the wild type, we reason that the reduced root growth in response to these two stresses is due to the AA deficiency in the mutant, because two other AA-deficient mutants, *vtc2-1* and *vtc4-1* show a similar response. Increased sensitivity of *vtc1-1* to salt stress due to low endogenous AA and impaired AA-glutathione cycle has been document previously (Huang et al., 2005).

Taken together, our data are consistent with previous reports that suggest a correlation between defective protein N-glycosylation, ER stress, and cell wall formation, cumulating in root growth arrest in *vtc1-1* in the presence of NH_4^+. However, we discovered distinct responses of *vtc1-1*, suggesting delineation between these secondary effects and yet unknown primary causes.

1.4.2 NH_4^+ sensitivity is pH-dependent

To obtain first insights into the primary cause of root growth inhibition in *vtc1-1*, we first tested the response of *vtc1-1* in comparison to the wild type grown on acidic, neutral and alkaline growth media containing NH_4^+. This was important to investigate, because the external pH influences cation uptake (Marschner, 1995). While alterations in the pH of the growth medium in the absence of NH_4^+ did neither have a significant effect on root growth in the wild type nor in *vtc1-1* (Fig. 1.7a), primary root length increased slightly in the wild type and dramatically in *vtc1-1* at neutral pH and decreased rapidly in both genotypes at alkaline pH in the presence of NH_4^+ (Fig. 1.7b). While the pH in the medium was not monitored over time, our results with the buffering agent MES are consistent with those in the absence of MES (cf. Fig. 1.8 and Fig. 1.7b), suggesting that the pH in the medium is relatively stable over the growth period of seven days. Therefore, the results will have to be interpreted with caution and may be explained as follows. NH_4^+ uptake by plant roots depends on its electrochemical gradient across the plasma membrane for passive transport through carriers or channels that regulate the transport. In addition, an electrochemical gradient of H^+ across the plasma membrane is maintained by H^+-ATPases, which pump H^+ from the cytoplasm, generating the driving force for active transport of NH_4^+ across the plasma membrane. At low pH, the efficiency of the H^+-ATPases or an increased re-entry of H^+ may decrease the electrochemical gradient across the plasma membrane, thereby reducing the driving force for NH_4^+ uptake (Kleiner, 1985; Marschner, 1995; Dyhr-Jensen and Brix, 1996). At NH_4^+ concentrations greater than 1 mM, transport of NH_4^+ and/or NH_3 is passive (Ullrich et al., 1984; Wang et al., 1993; Britto and Kronzucker, 2006). Thus, it appears more likely that the external pH affects NH_4^+ uptake by influencing the speciation of the ions. That is, with increasing pH the proportion of NH_3 (and NH_4OH) increases (numbers on abscissa in Fig. 1.7b, Table 1.3), resulting in an increased uptake through diffusion, as the plasma membrane is more permeable to uncharged solutes (Kleiner, 1985; Marschner, 1995). Therefore, we suggest that low pH may have an adverse effect on NH_4^+ uptake due to a combination of reduced H^+-ATPase activity, re-entry of H^+ and a lower amount of NH_3 that is liberated from NH_4^+ and presumably entering the cell where it is protonated again for assimilation and growth. At pH 7.0, which is close to the pH of the cytosol, H^+-ATPase activity is optimal, generating a sufficient gradient for NH_4^+ uptake and liberating a higher amount of NH_3 that can penetrate the plasma membrane and assimilated as NH_4^+. Note that *vtc1-1* root growth is dramatically enhanced at pH 7.0 compared to the acidic pH values. However, primary roots are still shorter in *vtc1-1* compared to the wild type (Fig. 1.7b). Finally, at alkaline pH, overall net NH_4^+ uptake is presumably decreased (Dyhr-Jensen and Brix, 1996), because larger amounts of NH_3 are freed in the medium, permeating the plasma membrane,

causing alkalinization of the cytosol, which in turn liberates NH_3 that is diffusing back out into the surrounding medium. In fact, it has been reported recently that root growth inhibition by NH_4^+ in Arabidopsis is mediated by enhanced NH_4^+ efflux (Li et al., 2010).

We suggest that, under conditions when NH_4^+ is the primary N source, the combination of lost N for assimilation and alkalization of the cytosol causes metabolic changes, leading to the drastic root growth defect in both genotypes (Fig. 1.7b). A more difficult question to answer is how the proline to serine mutation in *vtc1-1* affects these processes and thereby influences growth homeostasis.

1.4.3 Understanding the biochemical properties of GMPase protein will enhance our understanding of NH_4^+ sensitivity in plants

Arabidopsis GMPase has previously been suggested to localize to the cytosol (Wheeler et al., 1998; Conklin et al., 1999). Although we do not yet have protein biochemical data of the *Arabidopsis* GMPase in the presence and absence of NH_4^+ in the wild type and *vtc1-1* available, our results on *VTC1* transcript levels in the wild type, *vtc1-1* homozygous and *vtc1-1 VTC1* heterozygous plants (Fig. 1.12b) suggest a regulatory mechanism at the transcription level that is possibly mediated by the VTC1 protein. This could be an autoregulatory mechanism or through interaction with a regulatory partner. This mechanism is disabled in the *vtc1-1* homozygous mutant, while in the *vtc1-1 VTC1* heterozygote, the *vtc1-1* mutation exacerbates the defect. Thus, we propose that the *vtc1-1* mutation acts as a dominant-negative mutation and the mutated VTC1 protein product acts antagonistically to the wild-type allele. Dominant-negative mutations usually result in an altered molecular function and are characterized by a dominant or semi-dominant phenotype. Genetic and phenotypic analysis of *vtc1-1* demonstrated previously that *vtc1-1* is conferred by a semi-dominant monogenic mutation with ozone sensitivity and AA levels of *vtc1-1 VTC1* heterozygotes (grown on soil in the absence of NH_4^+ stress) being intermediate between the wild type and *vtc1-1* homozygous mutants (Conklin et al., 1996). Interestingly, root growth and AA levels are fully recovered to wild-type levels in the *vtc1-1 VTC1* heterozygote in the presence of NH_4^+ (Fig. 1.12a, Fig. 1.13). We speculate that Arabidopsis *VTC1* occurs in form of a multiprotein complex, in which substrate turnover from one protein to the next determines gene regulation of each monomer. This regulation could take place at the post-transcriptional level, mediated by mRNA degradation. One could argue that the pleiotropic responses exhibited by *vtc1-1* homozygous mutants are compensatory mechanisms to increase *VTC1* expression, while the presence of one wild type *VTC1* copy in *vtc1-1* mutants does not elicit pleiotropic effects and therefore *VTC1* mRNA levels are low. Consequently, our data suggest possible protein-protein interactions (among VTC1 proteins and of VTC1 with other proteins).

Structural information on plant GMPase is not currently available. However, the crystal structure of the thermophylic bacterium *Thermotoga maritima* has recently been solved (Pelissier et al., 2010). Bacterial GMPase appears to be dimeric, whereas eukaryotic GMPase can adopt oligomeric forms, as has been reported for *Leishmania mexicana* and *Leishmania major* (Davis et al., 2004; Perugini et al., 2005). Structural analysis of the *T. maritima* GMPase shows that two molecules associate into a dimer through a tail-to-tail arrangement of the C-terminal domains (Pelissier et al., 2010). The *vtc1-1* mutation represents a point mutation of amino acid 22 from a conserved proline to a serine (Conklin et al., 1999). Similarly, the point mutation in the *hsn1* mutant constitutes a change of a conserved glycine into a serine at amino acid position 11 (Qin et al., 2008). Both mutations lie in the active site of the enzyme, which is highly conserved (Conklin et al., 1999) and situated in a deep pocket located in the N-terminal domain according to the *T. maritima* structure (Pelissier et al., 2010). Work on the *L. mexicana* GMPase suggested that oligomerization is driven by non-covalent interactions and that oligomers dissociate at low protein concentrations, at low ionic strength, and at alkaline pH. In light of these published findings, we propose that *Arabidopsis* GMPase forms oligomers, which may be disrupted when the cytosolic pH changes from neutral to acidic or alkaline, a process that may be induced by the addition of NH_4^+. It is possible that the structural changes induced by the mutations in *vtc1-1* and *hsn1* alter the predicted active site loop region such that the amount of active oligomers and thus enzyme activity is reduced. It also remains to be investigated whether GMPase interacts with other proteins.

In conclusion, our results demonstrate that GDP-mannose deficiency is not the primary cause of conditional NH_4^+ sensitivity in *vtc1-1*. Instead, we propose that investigating the biochemical properties of GMPase will provide a full understanding of the NH_4^+ sensitivity in plants.

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2 CHAPTER 2: The novel *Arabidopsis thaliana svt2* suppressor of the ascorbic acid-deficient mutant *vtc1-1* exhibits phenotypic and genotypic instability

2.1 INTRODUCTION

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 (Qin et al., 2008; Barth et al., 2010; Li et al., 2010; Kempinski et al., 2011). 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 to identify *vtc1-1* suppressor mutants that could then be isolated and studied.

One of the suppressor mutants isolated in the M_0 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 had 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 L*er*. The most intriguing result was that a small percentage of *svt2* M_1 plants would produce offspring that had phenotypic and genotypic similarities to Col in the M_2 generation. Even more interesting was the fact that some of those Col revertants in the M_2 generation would produce progeny that exhibited phenotypic and genotypic *svt2* characteristics again in the M_3 generation.

Interestingly, phenotypic instability of *Arabidopsis* alleles affecting a disease resistance gene cluster has recently been reported (Yi and Richards, 2008). In their work, Yi and Richards reported that exposure to EMS or through the generation of different F_1 hybrids, phenotypic instability was induced in the *bal* and *cpr1* mutant alleles. The authors later proposed that the high phenotypic instability is caused by a genetic mechanism (Yi and Richards, 2009).

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*.

2.2 MATERIALS AND METHODS

2.2.1 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. L*er*-0 wild-type seed were obtained from The Arabidopsis Biological Resource Center. Plants were grown in Metromix 360 potting soil at 23°C at day and at 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 1x 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 (Kempinski et al., 2011). 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.

2.2.2 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 (Weigel and Glazebrook, 2002).

2.2.3 Ethyl methanesulfonate mutagenesis

Seed of homozygous *vtc1-1 Arabidopsis thaliana* (Col-0 background) was mutagenized with ethyl methanesulfonate as described (Figure 2.1; Weigel and Glazebrook, 2002). Approximately, 1200 M_0 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-tetrazoioum assay (Conklin et al., 2000). Additional suppressor mutants were isolated by pooling seeds generated from M_1 plants. Putative mutants were isolated and allowed to self-pollinate to obtain seed.

2.2.4 Pollen grain analysis and microscopy

Pollen was taken from 4.5-week-old flowering plants of Col-0 and L*er* wild type and *vtc1-1* and *svt2* M_2 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).

2.2.5 Genomic DNA isolation

Genomic DNA was isolated from rosette leaves following the protocol outlined in (Kotchoni et al., 2009). In case of genomic DNA isolation from *vtc1-1* seeds, a small amount of dried seeds was crushed and the extraction procedure described previously (Kotchoni et al., 2009) 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 2.1. PCR reactions were run on 1.0% agarose gels stained with ethidium bromide.

2.2.6 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 (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 described in Kempinski et al., 2011) which 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_Ts of each triplicate were averaged and plotted against 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: $E=10^{(1/-slope)}$.

Table 2.1 Forward (F) and reverse (R) sequences for primers used in analyzing the *VTC1* gene and for amplifying five Col/L*er* Insertion/Deletion (InDel) polymorphisms

Primer Name	5` 3`
VTC1 G1 F	AAA AAT TCG TTC TAG ATG GAT GCT
VTC1 G1 R	ATG GCT GTA AAT TGG AAG AGA T
VTC1 G2 F	GAA CCC TTG TCT CTA AAA TA
VTC1 G2 R	CAA ATC CCA TAA TCT GTT CC
VTC1 G3 F	CAA TTT TGC TTA CTT CTC T
VTC1 G3 R	TGG ATG CAA CCG ACA CAA AAC AAT
VTC1 G4 F	ACA TTT TTA GCA GCT GGT ATT GAG
VTC1 G4 R	AGG TAA GAA CTG GCA GAC TAA AG
VTC1 G5 F	TCG CTT GAG ACC ATT GAC T
VTC1 G5 R	GAG GCT TCC CCA CCG TGA GAT TTG
VTC1 G6 F	CAA GCT GGA AAT CAA AAT CAC T
VTC1 G6 R	GCG CTG CTG CAA TCT TAG G
VTC1 G7 F	ACA AAT CTC ACG GTG GGG AAG C
VTC1 G7 R	TGG TTA ATT TGG CAG GAG A
VTC1 G8 F	CAA GGG CTC TAT GCT ATG GTG
VTC1 G8 R	GCG TTT TGA TTG ATG CTT ATT C
VTC1 G9 F	GCG TGT ATC TCG AGC AGT ATC AT
VTC1 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

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; De Preter et al., 2002; Duarte et al., 2010). *VTC1* RQ was calculated from the average *VTC1* RQ of three biological replicates per genotype was normalized to the average RQ of *PAD4* from three replicates of each respective genotype, all run in the same reaction plate.

2.2.7 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.

2.2.8 Ascorbic acid quantification

To screen mutants, AA levels were analyzed qualitatively in small pieces of 2-week-old rosette leaves using the nitroblue tetrazolium assay described in (Conklin et al., 2000). The AA content was determined in whole rosettes of 3-week-old plants using the iron reduction assay (Mukherjee et al., 2010).

2.3 RESULTS

2.3.1 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 to decipher the pleiotropic phenotypes displayed by *vtc1-1*, including its hypersensitivity to ammonium (Qin et al., 2008; Barth et al., 2010; Li et al., 2010; Kempinski et al., 2011). We employed a gene suppressor analysis with the goal to identify novel genes that interact or regulate *VTC1*. Seed of the *vtc1-1* mutant, which is in the Col-0 genetic background (Conklin et al., 1996), were subjected to chemical mutagenesis using EMS (Weigel and Glazebrook, 2002). 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 test (Conklin et al., 2000). 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; Fig. 2.1). Note that we isolated additional suppressor mutants by pooling M_2 seed and screening for long roots on 1x 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 all had long roots, whereas the other five lines segregated in a ratio of three plants producing long roots, one plant having short roots. All of these suppressor mutants had a Col-like phenotype

and lacked the *vtc1-1* mutation (Kempinski et al., unpublished results). Except for root development assessment, these suppressor mutants were not yet characterized further.

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

The first observation we made when characterizing *svt2* M_1 plants was that *svt2* exhibited a phenotype reminiscent of the L*er* ecotype with the characteristic round leaves compared to Col. Yet, *svt2* had features that were distinct from the L*er* phenotype. The *svt2* mutant displayed the characteristic erect morphology that is typical for L*er*-0 (Fig. 2.2a). However, *svt2* had an overall enlarged morphology as is evident in the larger rosettes (inset of 3-week-old rosettes in Fig. 2.2A) and flowers (inset, Fig. 2.2a). In addition, *svt2* was strongly delayed in flowering compared to the Col-0 and L*er*-0 wild types and the *vtc1-1* mutant (Fig 2.2a). The flowering data are consistent with previous reports, with L*er*-0 wild type entering the reproductive phase before Col-0 wild type. An early flowering phenotype of *vtc1-1* has been reported previously (Kotchoni et al., 2009).

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 L*er*-0 wild-types and *vtc1-1* and *svt2* mutants. As is shown in Fig. 2.2B, 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 L*er*-0 wild types and in the *vtc1-1* and *svt2* mutants (Table 2.1). 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.

The AA content in *svt2* was similar to levels quantified in Col-0 and L*er*-0 wild types, whereas *vtc1-1* contained only 30% of the AA content as expected (Fig. 2.2c; Conklin et al., 1996). Finally, we investigated whether *svt2* also exhibits recovered root development in the presence of ammonium by growing the four genotypes in full strength 1x MS medium. The *vtc1-1* mutant is conditionally hypersensitive to ammonium (Qin et al., 2008; Barth et al., 2010; Kempinski et al., 2011). Figure 2d 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.

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.

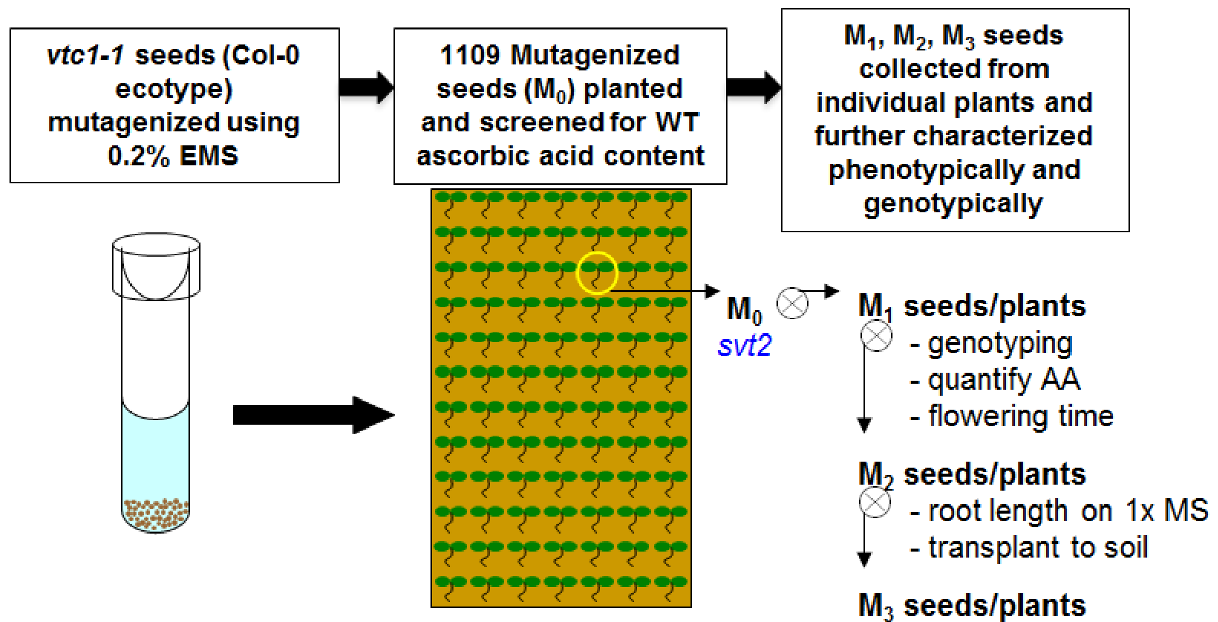

Figure 2.1 Schematic of the isolation of the *vtc1-1* suppressor mutant *svt2*.
Homozygous *vtc1-1* seed (in the Col-0 genetic background), were exposed to chemical mutagenesis using ethyl methanesulfonate (EMS). Over one thousand mutagenized seed (M_0) were planted on soil and screened for wild-type levels of ascorbic acid. The only mutant isolated in the M_0 generation containing recovered a ascorbic acid content was *svt2*. The mutant was allowed to self-fertilize and was characterized phenotypically and genotypically in subsequent generations.

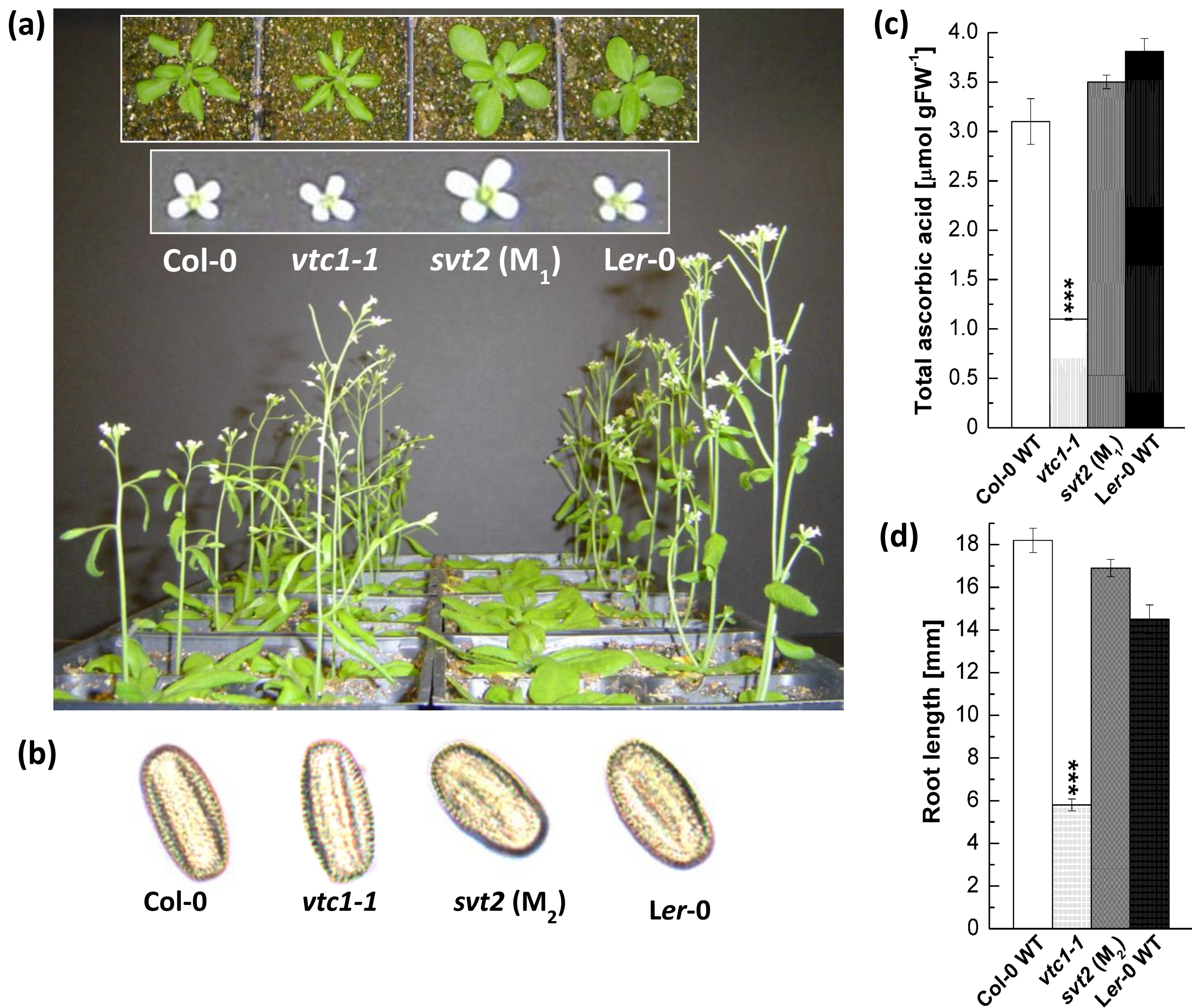

Figure 2.2 Phenotypic characterization of *svt2*.

(a) Flowering phenotype of four-week-old Col-0 wild type, the *vtc1-1* and *svt2* mutants and the L*er*-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) Pollen phenotype of the four genotypes when plants were 4.5 weeks old. (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 1x MS. Bars represent mean ± SE of 30-90 individuals. *** $P < 0.001$ by Student's *t*-test indicates significant differences.

2.3.3 *svt2* shares genome similarity with L*er*

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 primer sets (Table 2.1) and PCR-amplified the entire *VTC1* gene including approximately 500 bp of the promoter region directly upstream of the first base in the 5' UTR in genomic DNA extracted from Col-0 and L*er*-0 wild types, and *vtc1-1* and *svt2* mutants (Fig. 2.3a). 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 (Fig. 2.4). The G1F/G1R primer set, used to amplify the *VTC1* promoter region (Fig. 2.3a), generated a larger PCR product in *svt2* than in the wild type. 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 (Fig. 2.3a). 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 L*er*-0 wild type (Fig. 2.3b), suggesting a L*er* insertion polymorphism. Thus, these data imply that *svt2* shares both phenotypic and genotypic similarities with L*er*.

We therefore assessed five additional Insertion/Deletion (InDel) polymorphisms randomly chosen across the five *Arabidopsis* chromosomes (Table 2.1) in *svt2* compared to the Col-0 and L*er*-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 L*er*-0 genomic DNA (Fig. 2.5). Moreover, sequence analysis of the *VTC1* gene and promoter region revealed that *svt2* contained a 280 bp insertion in the *VTC1* promoter (Fig. 2.3c); DNA sequence highlighted in gray in Fig. 2.6) in addition to other single nucleotide polymorphisms (see examples indicated by upright arrows in Figs. 2.3c, 2.6). 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* L*er* GenBank sequence, the *VTC1* gene sequence in *svt2* shared sequence similarities with L*er* (upright arrows in Figs. 2.3c, 2.6) and Col (see arrows pointing down in Fig. 2.6). However, note that there are sequences that are unique to *svt2* and are not shared between Col, *vtc1-1* or L*er* (see arrow heads in Fig. 2.6). Finally, note the overlap in sequences between Col, *vtc1-1*, *svt2* and L*er* on the 5' end of the sequence flanking the

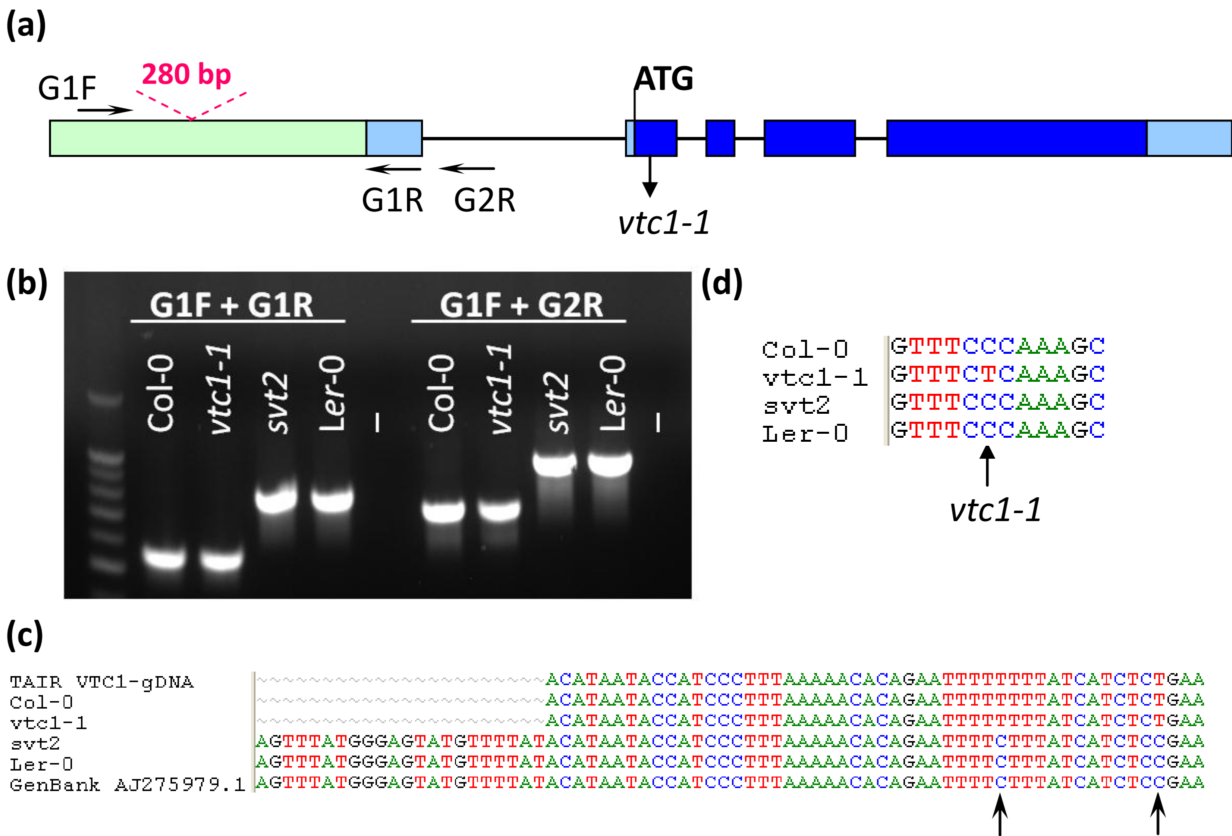

Figure 2.3 Genotypic characterization of *svt2*.

(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 280 bp in L*er*-0 *VTC1*. (b) PCR amplification of the *VTC1* promoter region in the Col-0 wild type, *vtc1-1* and *svt2* mutants and L*er*-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 L*er*-0 wild type and the GenBank sequence. Arrows indicate single nucleotide polymorphisms between the L*er*-0 and Col-0 sequence. (d) Point mutation in *vtc1-1*, a conversion from a cytosine to a thymine.

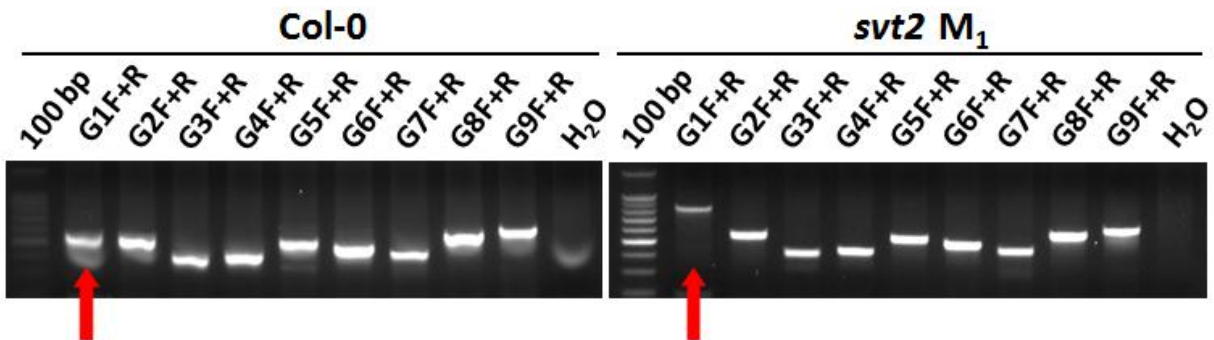

Figure 2.4 Molecular characterization of *svt2*.
Amplification of the *VTC1* gene including approximately 500 bp of the promoter region using a series of nine, overlapping primers (G1F+R through G9F+R) in both Col-0 wild type and *svt2* M_1 mutant genomic DNA. The last lane in each gel contained a negative control (water instead of DNA). Red arrows indicate the different sized PCR products using the same primer set.

Table 2.2 Quantitative PCR to verify that *VTC1* is a single copy gene in Col-0 and L*er*-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	
	VTC1/PAD4	
Col-0 WT	0.3796	**VTC1 E=1.8**
vtc1-1	0.5843	
svt2	0.5504	
L*er*-0 WT	0.6329	
Col-0	0.3153	**VTC1 E=2**
vtc1-1	0.5292	
svt2	0.4946	
L*er*-0 WT	0.5807	

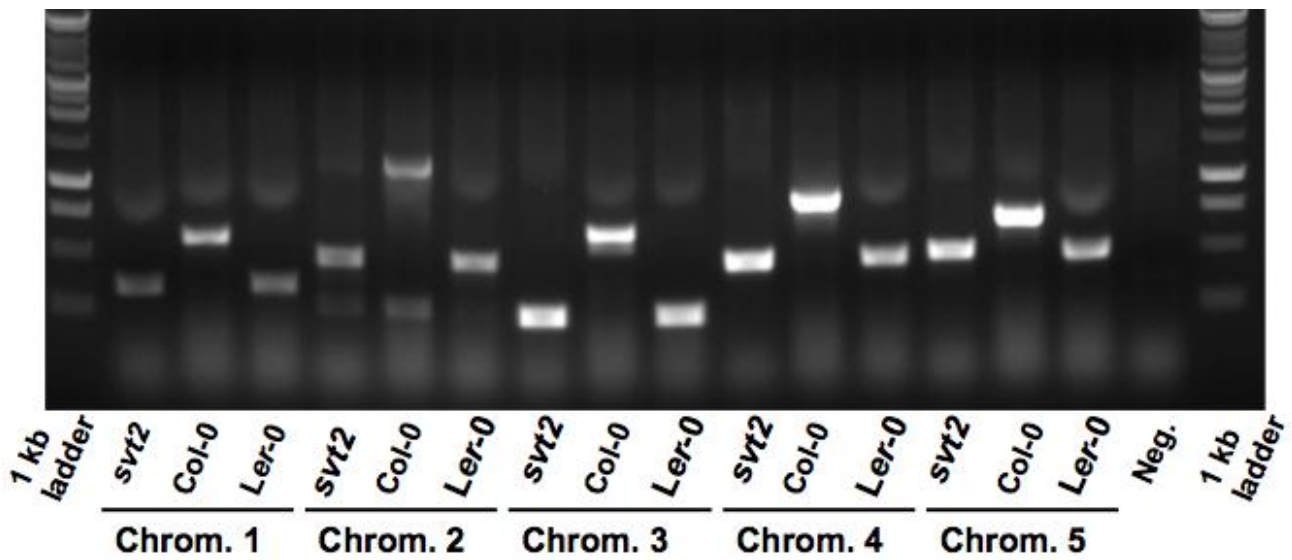

Figure 2.5 Insertion/Deletion polymorphism analysis in *svt2*, Col-0 and L*er*-0.
Primers were designed for five randomly selected InDel polymorphisms across the five *Arabidopsis* chromosomes. The polymorphisms represent insertions in Col-0 and deletions in L*er*.

insertion (at approximately base pair 1902; see horizontal black arrows in Fig. 2.6) compared to the sequence flanking the 3’ end of the DNA sequence insertion (starting at base pair 2273; see horizontal black arrows in Fig. 2.6).

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 (Figs. 2.3d, 2.6).

In summary, our data demonstrate that *svt2* shares DNA sequence similarity with Col and L*er*, 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 L*er*, we analyzed subsequent *svt2* generations and discovered additional characteristics that are unique to *svt2*.

2.3.4 *svt2* exhibits phenotypic and genotypic instability

Our initial observations revealed that approximately 10% of *svt2* M_2 plants displayed a Col-like phenotype. Therefore, we planted *svt2* M_1, M_2, and M_3 progeny to check whether this result can be repeated and to determine the segregation ratio (Table 2.3). Additionally, we investigated whether phenotypic Col-like revertants would produce *svt2* (L*er*-like) offspring in the next generation.

As summarized in Table 2.3, revertants could only be detected when a relatively large population was planted. In the *svt2* M_1 generation, only 1% of Col-like revertants were detected. In contrast, 8%-10% of *svt2* M_2 plants displayed a Col-like phenotype, whereas no revertants were detected in the *svt2* M_3 generation. These Col-like revertants were isolated and seeds were collected from individual plants and the phenotype of the progeny in the M_3 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 M_2 generation, their M_3 progeny continued to appear as Col-like plants. This was the case for the M_3 progeny of the A8 and G7 plants listed in Table 2.4 However, out of 63 progeny from the K1 revertant plant, one reverted back to a *svt2*-like phenotype (Table 2.3). The K1 double revertant switched from *svt2* phenotype in the M_1 generation to a Col-like phenotype in the M_2 generation, and then back to a *svt2*-like phenotype in the M_3 generation. Note that only a small number of progeny was planted. In case of the *svt2* Col R1 revertant, 20 individuals displaying a *svt2*-like phenotype were observed (Table 2.3).

```
                                 10        20        30        40        50        60
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     ACGTCGCTATATATTGAAAGCATGTTTGACTATGTTACGTTAGCTATCATTTTATAAATG
vtc1-1_VTC1_gDNA         ACGTCGCTATATATTGAAAGCATGTTTGACTATGTTACGTTAGCTATCATTTTATAAATG
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                 70        80        90       100       110       120
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     ACGTGATGATTACGAATTTACGATATACGTACTGCTTAAAGAAGAAGAATGTAAGAAGAA
vtc1-1_VTC1_gDNA         ACGTGATGATTACGAATTTACGATATACGTACTGCTTAAAGAAGAAGAATGTAAGAAGAA
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                130       140       150       160       170       180
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     AAATCTATTGGCGCAAAACGTCGGTGAGTCAAAGCTGAAATCAAGGTGCTGCCTAACTAC
vtc1-1_VTC1_gDNA         AAATCTATTGGCGCAAAACGTCGGTGAGTCAAAGCTGAAATCAAGGTGCTGCCTAACTAC
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                190       200       210       220       230       240
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     GATGTTGCTTTGGTATCTTTCCATAACTTTCTCTTTTTGTGACTTTTTTTTTTTTTTTTT
vtc1-1_VTC1_gDNA         GATGTTGCTTTGGTATCTTTCCATAACTTTCTCTTTTTGTGACTTTTTTTTTTTTTTTTT
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                250       260       270       280       290       300
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     TTTCATTTCTAAATTCCTTCATTTAACTTTTCTAAAGTATTGTAGTATTACTTTAAAATC
vtc1-1_VTC1_gDNA         TTTCATTTCTAAATTCCTTCATTTAACTTTTCTAAAGTATTGTAGTATTACTTTAAAATC
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                310       320       330       340       350       360
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     AACCAAAATTTATACAGTATTGTTACTATGATGTGTGTATGCATATGGGGTCACCGCGTT
vtc1-1_VTC1_gDNA         AACCAAAATTTATACAGTATTGTTACTATGATGTGTGTATGCATATGGGGTCACCGCGTT
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                370       380       390       400       410       420
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     TGATGGTGGCGTGTATTTGTCAAAATGGACTATGCTTTTAATTAAACGTTAGATCTGGGG
vtc1-1_VTC1_gDNA         TGATGGTGGCGTGTATTTGTCAAAATGGACTATGCTTTTAATTAAACGTTAGATCTGGGG
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                430       440       450       460       470       480
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     TTCTCTATTCTTCTTTTTTAGTCATAAATCATAATATATACGTTATGATATAATATTGTA
vtc1-1_VTC1_gDNA         TTCTCTATTCTTCTTTTTTAGTCATAAATCATAATATATACGTTATGATATAATATTGTA
svt2_VTC1_gDNA           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                490       500       510       520       530       540
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     GCCTCTATGTTTCGATTTTTGTTCCTCGATGTATCAATCTTGAAAAGAATCTTAGTTTCA
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vtc1-1_VTC1_gDNA           GCCTCTATGTTTCGATTTTTGTTCCTCGATGTATCAATCTTGAAAAGAATCTTAGTTTCA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    550       560       570       580       590       600
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       TTCTAGGTAATCTCAAGGTGTGAAAATCCTATGTACATAAATATGTAAAGAATTCTCAAC
vtc1-1_VTC1_gDNA           TTCTAGGTAATCTCAAGGTGTGAAAATCCTATGTACATAAATATGTAAAGAATTCTCAAC
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    610       620       630       640       650       660
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       CATTATTCTTAAACCAAAAATCTCTATCAAAACCGGTCCAACACCAGAACATCTTGATGA
vtc1-1_VTC1_gDNA           CATTATTCTTAAACCAAAAATCTCTATCAAAACCGGTCCAACACCAGAACATCTTGATGA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    670       680       690       700       710       720
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       TCTAGACAAATCAGTTTCTTAAAAAAATTATTGATATGCACCACTTTACTAAGAAGGAGA
vtc1-1_VTC1_gDNA           TCTAGACAAATCAGTTTCTTAAAAAAATTATTGATATGCACCACTTTACTAAGAAGGAGA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    730       740       750       760       770       780
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       ATAACAAAAAAAACAAAATGAAGGTACCTTGTCGGTGTCACGTGGGGAAGAACATGTGAA
vtc1-1_VTC1_gDNA           ATAACAAAAAAAACAAAATGAAGGTACCTTGTCGGTGTCACGTGGGGAAGAACATGTGAA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    790       800       810       820       830       840
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       AACAAACGAACAATAATTGGAAGAAAAAAAAAATGTGAATCTTGTGATGGATCAAATTAGG
vtc1-1_VTC1_gDNA           AACAAACGAACAATAATTGGAAGAAAAAAAAAATGTGAATCTTGTGATGGATCAAATTAGG
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    850       860       870       880       890       900
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       TATATTCTTTGTAGCTCTTTAGTGTTCGTTCACTCACAGCCTCAGGCCCTCAGCTTTGGT
vtc1-1_VTC1_gDNA           TATATTCTTTGTAGCTCTTTAGTGTTCGTTCACTCACAGCCTCAGGCCCTCAGCTTTGGT
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    910       920       930       940       950       960
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       CTTATCCAAACGACTGGCCCGCATAACAGAGACATTACACAATACGAATGCGTTTCTATA
vtc1-1_VTC1_gDNA           CTTATCCAAACGACTGGCCCGCATAACAGAGACATTACACAATACGAATGCGTTTCTATA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                    970       980       990      1000      1010      1020
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       TTACTGCTCCATACTCAAATCGAATCCTGGAGACAGATTCTTATTTTTTTTGTTATATTC
vtc1-1_VTC1_gDNA           TTACTGCTCCATACTCAAATCGAATCCTGGAGACAGATTCTTATTTTTTTTGTTATATTC
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

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                                        1030      1040      1050      1060      1070      1080
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          AATTGTTTAGATTACTAAAAGCCAAAATCATAAAAACTTTAAACGATTTGATTATTATAT
vtc1-1_VTC1_gDNA              AATTGTTTAGATTACTAAAAGCCAAAATCATAAAAACTTTAAACGATTTGATTATTATAT
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1090      1100      1110      1120      1130      1140
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          CACCAAAATATTGGCTTTTAATCCATGTTAATACTGATTAACTTGAAATATACTTATATT
vtc1-1_VTC1_gDNA              CACCAAAATATTGGCTTTTAATCCATGTTAATACTGATTAACTTGAAATATACTTATATT
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1150      1160      1170      1180      1190      1200
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          TTTGGTTATTGGGGAGGAAGGAATACAAATAATCTTTTTCGACACTGAATAGTTTCCTTT
vtc1-1_VTC1_gDNA              TTTGGTTATTGGGGAGGAAGGAATACAAATAATCTTTTTCGACACTGAATAGTTTCCTTT
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1210      1220      1230      1240      1250      1260
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          GAATGAAAGCTGATAAAATAGTGTATAAGTTCATTTTTTTATAACGTAATTTTTTTTTTG
vtc1-1_VTC1_gDNA              GAATGAAAGCTGATAAAATAGTGTATAAGTTCATTTTTTTATAACGTAATTTTTTTTTTG
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1270      1280      1290      1300      1310      1320
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          GTAATGTGAACACCCTAAAAAGATCTCCTTCTGATGTAGAAATATTATACCCATACACAT
vtc1-1_VTC1_gDNA              GTAATGTGAACACCCTAAAAAGATCTCCTTCTGATGTAGAAATATTATACCCATACACAT
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1330      1340      1350      1360      1370      1380
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          CGATAAGTTAGTTTGTGGAAAGAAAAACTAACTAACAAAATAACACCTTCAATTCGACAA
vtc1-1_VTC1_gDNA              CGATAAGTTAGTTTGTGGAAAGAAAAACTAACTAACAAAATAACACCTTCAATTCGACAA
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1390      1400      1410      1420      1430      1440
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          AAGAGCATAAGTTTAATAAAATACCTACAGAAATTTTTGACGATGATACCACTACCATTC
vtc1-1_VTC1_gDNA              AAGAGCATAAGTTTAATAAAATACCTACAGAAATTTTTGACGATGATACCACTACCATTC
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1450      1460      1470      1480      1490      1500
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          GCCAAAACCACAATAATTCAATTCTTCTAAATGTAGATTCTATTAATATGGTCGATTGTC
vtc1-1_VTC1_gDNA              GCCAAAACCACAATAATTCAATTCTTCTAAATGTAGATTCTATTAATATGGTCGATTGTC
svt2_VTC1_gDNA                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        1510      1520      1530      1540      1550      1560
                              ....|....|....|....|....|....|....|....|....|....|....|....|
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Col-0_VTC1_TAIR_gDNA       ATTATTCCAAATAATTTATTTTTTTAAATGAAAAATTCATAACTCTAAATATACAAAAGC
vtc1-1_VTC1_gDNA           ATTATTCCAAATAATTTATTTTTTTAAATGAAAAATTCATAACTCTAAATATACAAAAGC
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                     1570      1580      1590      1600      1610      1620
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       AATTGCTGATTTGGATTCAAATTTTCAAAGAGAACTTGTAGTTATGCATATACATAAAAA
vtc1-1_VTC1_gDNA           AATTGCTGATTTGGATTCAAATTTTCAAAGAGAACTTGTAGTTATGCATATACATAAAAA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                     1630      1640      1650      1660      1670      1680
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       GGTAAGTCAGTTTTTTTTTTTGGCTAATAAAAACGGTAAAATCATGTTATTGATAAAAAA
vtc1-1_VTC1_gDNA           GGTAAGTCAGTTTTTTTTTTTGGCTAATAAAAACGGTAAAATCATGTTATTGATAAAAAA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                     1690      1700      1710      1720      1730      1740
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       TTTGAAAACAGTAAAAGAAATATGACCATATTTGAAAATTTACCTAAAAACTATAATGAT
vtc1-1_VTC1_gDNA           TTTGAAAACAGTAAAAGAAATATGACCATATTTGAAAATTTACCTAAAAACTATAATGAT
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                     1750      1760      1770      1780      1790      1800
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       GTAATAGTTTGATTTACTTACATTTAACTAATTTTTAAGGTCTGTTGATCGAACTCGTTA
vtc1-1_VTC1_gDNA           GTAATAGTTTGATTTACTTACATTTAACTAATTTTTAAGGTCTGTTGATCGAACTCGTTA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~TCT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                     1810      1820      1830      1840      1850      1860
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       CCTTGAACAATTAGTAGGACAACTCAAACCACTAAACCATTATACTTTAAGGATTTATGT
vtc1-1_VTC1_gDNA           CCTTGAACAATTAGTAGGACAACTCAAACCACTAAACCATTATACTTTAAGGATTTATGT
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                     1870      1880      1890      1900      1910      1920
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       AAAATTTCAAATATATATAGTTTAGTAGATGCACTTATCATCACACTCACCAATTGGATG
vtc1-1_VTC1_gDNA           AAAATTTCAAATATATATAGTTTAGTAGATGCACTTATCATCACACTCACCAATTGGATG
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                     1930      1940      1950      1960      1970      1980
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       TCAACACCTGGTTCTAGCTTTTTAATTACCAAAGTGAAAAAACTGACTTTTTCTAAAAAA
vtc1-1_VTC1_gDNA           TCAACACCTGGTTCTAGCTTTTTAATTACCAAAGTGAAAAAACTGACTTTTTCTAAAAAA
svt2_VTC1_gDNA             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_GenBank_gDNA    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                               <--------
                                     1990      2000      2010      2020      2030      2040
                           ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA       AATTCGTTCTAGATGGATGCTCTTCAAATTCGTTC~~TAAAAAAAACTG~~~~~~~~~~~
vtc1-1_VTC1_gDNA           AATTCGTTCTAGATGGATGCTCTTCAAATTCGTTC~~TAAAAAAAACTG~~~~~~~~~~~
svt2_VTC1_gDNA             ~~~~~~~~~~~~ATGGATGCTCTTTAAAT C TTCGATATTTTTTATCCGTTTCGATAAT
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Ler-0_VTC1_GenBank_gDNA     ~~~~~~~~~~AGATGGATGCTCTTTAAAT~C~TTCGATATTTTTTATCCGTTTCGATAAT

                                      2050      2060      2070      2080      2090      2100
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
vtc1-1_VTC1_gDNA            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2_VTC1_gDNA              ATGGTAAGAATGAATGACGAATCGGTCAAGCTAATCTGTATATTAATCATTGTACTCATC
Ler-0_VTC1_GenBank_gDNA     ATGGTAAGAATGAATGACGAATCGGTCAAGCTAATCTGTATATTAA~CATTGTACTCATC

                                      2110      2120      2130      2140      2150      2160
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
vtc1-1_VTC1_gDNA            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2_VTC1_gDNA              AACGTAAAGTCCTATTCGTCTATACATATGTGAACTTATATATGTCTATCAACTAGTTCA
Ler-0_VTC1_GenBank_gDNA     AACGTAAAGTCCTATTCGTCTATACATATGTGAACTTATATATGTCTATCAACTAGTTCA

                                      2170      2180      2190      2200      2210      2220
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
vtc1-1_VTC1_gDNA            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2_VTC1_gDNA              CTACCCTATATATAAAGTTCATCAGAGTTGTCGATCAGCAGTGACCACTACACATTCTTC
Ler-0_VTC1_GenBank_gDNA     CTACCCTAT~~~~AAAGTTCATCAGAGTTGTCGATCAGCAGTGACCACTACACATTCTTC

                                      2230      2240      2250      2260      2270      2280
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ACATAATA
vtc1-1_VTC1_gDNA            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ACATAATA
svt2_VTC1_gDNA              ATACAGCTGAGTTAGGAATGTTAACAAAATAGTTTATGGGAGTATGTTTTATACATAATA
Ler-0_VTC1_GenBank_gDNA     ATACAGCTGAGTTAGGAATGTTAACAAAATAGTTTATGGGAGTATGTTTTATACATAATA

                                      2290      2300      2310      2320      2330      2340
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        CCATCCCTTTAAAAACACAGAATTTTTTTTATCATCTCTGAAACAAATCATTTACAGTAG
vtc1-1_VTC1_gDNA            CCATCCCTTTAAAAACACAGAATTTTTTTTATCATCTCTGAAACAAATCATTTACAGTAG
svt2_VTC1_gDNA              CCATCCCTTTAAAAACACAGAATTTTCTTTATCATCTCCGAAACAAATCATTTACAGTAG
Ler-0_VTC1_GenBank_gDNA     CCATCCCTTTAAAAACACAGAATTTTCTTTATCATCTCCGAAACAAATCATTTACAGTAG

                                      2350      2360      2370      2380      2390      2400
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        TAAATGT~~CAACACAACATTAATTCTGTTTGTTGTTGGCATTTACAATTGCAAAATCAT
vtc1-1_VTC1_gDNA            TAAATGT~~CAACACAACATTAATTCTGTTTGTTGTTGGCATTTACAATTGCAAAATCAT
svt2_VTC1_gDNA              TAAATGTAAAAACACAACATTAATTCTGTTTGTTGTTGGCATTTACAATTGCAAAATCAT
Ler-0_VTC1_GenBank_gDNA     TAAATGTAAAAACACAACATTAATTCTGTTTGTTGTTGGCATTTACAATTGCAAAATCAT

                                      2410      2420      2430      2440      2450      2460
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        TTTCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAATATTCATAG
vtc1-1_VTC1_gDNA            TTTCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAATATTCATAG
svt2_VTC1_gDNA              TTTCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAATATTCATAG
Ler-0_VTC1_GenBank_gDNA     TTTCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAATATTCATAG

                                      2470      2480      2490      2500      2510      2520
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        AAAAAGAAAAGAGCCATTAATTAATGGCTTGAAGAAAGATTGGTGTATAAGCGTCTACGT
vtc1-1_VTC1_gDNA            AAAAAGAAAAGAGCCATTAATTAATGGCTTGAAGAAAGATTGGTGTATAAGCGTCTACGT
svt2_VTC1_gDNA              AAAAA~AAAAGAGCCATTAATTAATGGCTTGAAGAAAGATTGGTGTATAAGCGTCTACGT
Ler-0_VTC1_GenBank_gDNA     AAAAA~AAAAGAGCCATTAATTAATGGCTTGAAGAAAGATTGGTGTATAAGCGTCTACGT

                                      2530      2540      2550      2560      2570      2580
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                             ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA         GACCTTTAATTAATTTACTTCCCCCAAAAAAGTCAACATTCAACATGTGAATAAAAA~TC
vtc1-1_VTC1_gDNA             GACCTTTAATTAATTTACTTCCCCCAAAAAAGTCAACATTCAACATGTGAATAAAAA~TC
svt2_VTC1_gDNA               GACCTTTAATTAATTTACTTCCCCAAAAAAAGTCAACATTCAACATGTGAATAAAAA~TC
Ler-0_VTC1_GenBank_gDNA      GACCTTTAATTAATTTACTTCCCCAAAAAAAGTCAACATTCAACATGTGAATAAAAAATC

                                     2590      2600      2610      2620      2630      2640
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA         AATATTGGTTTCTAAGTAAGTAAGTACCATATTATTAAATTATTTATTTTGGTAAATACG
vtc1-1_VTC1_gDNA             AATATTGGTTTCTAAGTAAGTAAGTACCATATTATTAAATTATTTATTTTGGTAAATACG
svt2_VTC1_gDNA               AATATTGGTTTCTAAGTAAGTAAGTACCATATTATTAAATTATTTATTTTGGTAAATACG
Ler-0_VTC1_GenBank_gDNA      AATATTGGTTTCTAAGTAAGTAAGTACCATATTATTAAATTATTTATTTTGGTAAATACG

                                     2650      2660      2670      2680      2690      2700
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA         CACTCAATTTTTCTCTCAACGGTG~~~~TATATAAACAAAAGGAGTCTCCTTTGGAAAAA
vtc1-1_VTC1_gDNA             CACTCAATTTTTCTCTCAACGGTG~~~~TATATAAACAAAAGGAGTCTCCTTTGGAAAAA
svt2_VTC1_gDNA               CACTCAATTTTTCTCTCAACGGTGGGTGTATATAAACAAAAGGAGTCTCCTTTGGAAAAA
Ler-0_VTC1_GenBank_gDNA      CACTCAATTTTTCTCTCAACGGTGGGTGTATATAAACAAAAGGAGTCTCCTTTGGAAAAA

                                     2710      2720      2730      2740      2750      2760
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA         ACTTGCCTATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCTGACGC
vtc1-1_VTC1_gDNA             ACTTGCCTATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCTGACGC
svt2_VTC1_gDNA               ACTTGCCTATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCTGACGC
Ler-0_VTC1_GenBank_gDNA      ACTTGCCTATCATTTTGCCAACGAACGTTCTTTCTTCTTAATCACAGCTCAGCCT~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         GTGAGATCTCTCTCAAGGTAATGCCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG
vtc1-1_VTC1_gDNA             GTGAGATCTCTCTCAAGGTAATGCCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG
svt2_VTC1_gDNA               GTGAGATCTCTCTCAAGGTAATGCCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG
Ler-0_VTC1_GenBank_gDNA      GTGAGATCTCTCTCAAGGTAATGCCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATG

                                     2890      2900      2910      2920      2930      2940
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA         CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCTCATTTTGTATTTGCTGTTGTT
vtc1-1_VTC1_gDNA             CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCTCATTTTGTATTTGCTGTTGTT
svt2_VTC1_gDNA               CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCTCATTTTGTATTTGCTGTTGTT
Ler-0_VTC1_GenBank_gDNA      CATGTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCTCATTTTGTATTT~CTGTTGTT

                                     2950      2960      2970      2980      2990      3000
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA         GGTTTTTAATTCGATTTTCCGGAACAGATTATGGGATTT~GTATTCGAATCTTCGATTTG
vtc1-1_VTC1_gDNA             GGTTTTTAATTCGATTTTCCGGAACAGATTATGGGATTT~GTATTCGAATCTTCGATTTG
svt2_VTC1_gDNA               GGTTTTTAATTCGATTTTCCGGAACAGGTTATGGGGATTTGTATTCGAATCTTCGATTTG
Ler-0_VTC1_GenBank_gDNA      GGTTTTTAATTCGATTTTCCGGAACAGGTTATGGGGATTTGTAT~CGAATCTTCGATTTG

                                     3010      3020      3030      3040      3050      3060
                             ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA         ATGACATAATGTCCCAGCCTTTTATGTTTAATCTTGAAATGATGGACTTTTATCCGATCT
vtc1-1_VTC1_gDNA             ATGACATAATGTCCCAGCCTTTTATGTTTAATCTTGAAATGATGGACTTTTATCCGATCT
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svt2_VTC1_gDNA                ATGACATAATGTCCCAGCCATTTATGTTTAATCTTGAAATGATGGACTTTAATCCGATCT
Ler-0_VTC1_GenBank_gDNA       ATGACATAATGTCCCAGCCATTTATGTTTAATCTTGAAATGATGGACTTTAATCCGATCT

                                        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          TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGTCTGAATTTTGGAAAGAACTATT
vtc1-1_VTC1_gDNA              TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGTCTGAATTTTGGAAAGAACTATT
svt2_VTC1_gDNA                TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGTCTGAATTTTGGAAAGAACTATT
Ler-0_VTC1_GenBank_gDNA       TCCAACATTTTTAGCAGCTGGTATTGAGCTCTTGTTGTCTGAATTTTGGAAAGAACTATT

                                        3190      3200      3210      3220      3230      3240
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTCACCTTTTTCTCTGATTATT
vtc1-1_VTC1_gDNA              TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTCACCTTTTTCTCTGATTATT
svt2_VTC1_gDNA                TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTCACCTTTTTCTCTGATTATT
Ler-0_VTC1_GenBank_gDNA       TTTGTTGTATCGTTTTGATTTATTTGGATCTGAATTCATTCACCTTTTTCTCTGATTATT

                                        3250      3260      3270      3280      3290      3300
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          GTTTTGTGTCGGTTGCATCCACTTTGATTAGATCTGAATGAATCATTTTTTTATGTGCTC
vtc1-1_VTC1_gDNA              GTTTTGTGTCGGTTGCATCCACTTTGATTAGATCTGAATGAATCATTTTTTTATGTGCTC
svt2_VTC1_gDNA                GTTTTGTGTCGGTTGCATCCACTTTGATTAGATCTGAATGAATCATTTTTTTATGTGCTC
Ler-0_VTC1_GenBank_gDNA       GTTTTGTGTCGGTTGCATCCACTTTGATTAGATCTGAATGAATCATTTTTTTATGTGCTC

                                        3310      3320      3330      3340      3350      3360
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          AAGTTATTGTATGGATTGTTCTGTTTCTAGCATGTTTTGGTTAGACATTGTTAAGATCTG
vtc1-1_VTC1_gDNA              AAGTTATTGTATGGATTGTTCTGTTTCTAGCATGTTTTGGTTAGACATTGTTAAGATCTG
svt2_VTC1_gDNA                AAGTTATTGTATGGATTGTTCTGTTTCTAGCATGTTTTGGTTAGACATTGTTAAGATCTG
Ler-0_VTC1_GenBank_gDNA       AAGTTATTGTATGGATTGTTCTGTTTCTAGCATGTTTTGGTTAGACATTGTTAAGATCTG

                                        3370      3380      3390      3400      3410      3420
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          ACGTTTGCATTTTCAGGAAAAGGAG~TTAGAGCATCATCAAGATGAAGGCACTCATTCTT
vtc1-1_VTC1_gDNA              ACGTTTGCATTTTCAGGAAAAGGAG~TTAGAGCATCATCAAGATGAAGGCACTCATTCTT
svt2_VTC1_gDNA                ACGTTTGCATTTTCAGGAAAAGGAG~TTAGAGCATCATCAAGATGAAGGCACTCATTCTT
Ler-0_VTC1_GenBank_gDNA       ACGTTTGCATTTTCAGGAAAAGGAGCTTAGAGCATCATCAAGATGAAGGCACTCATTCTT

                                        3430      3440      3450      3460      3470      3480
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          GTTGGAGGCTTCGGCACTCGCTTGAGACCATTGACTCTCAGTTTCCCAAAGCCCCTTGTT
vtc1-1_VTC1_gDNA              GTTGGAGGCTTCGGCACTCGCTTGAGACCATTGACTCTCAGTTTCTCAAAGCCCCTTGTT
svt2_VTC1_gDNA                GTTGGAGGCTTCGGCACTCGCTTGAGACCATTGACTCTCAGTTTCCCAAAGCCCCTTGTT
Ler-0_VTC1_GenBank_gDNA       GTTGGAGGCTTCGGCACTCGCTTGAGACCATTGACTCTCAGTTTCCCAAAGCCCCTTGTT

                                        3490      3500      3510      3520      3530      3540
                              ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA          GATTTTGCTAATAAACCCATGATCCTTCATCAGGTAATCTATCTTAAATTTGCCGCTTTA
vtc1-1_VTC1_gDNA              GATTTTGCTAATAAACCCATGATCCTTCATCAGGTAATCTATCTTAAATTTGCCGCTTTA
svt2_VTC1_gDNA                GATTTTGCTAATAAACCCATGATCCTTCATCAGGTAATCTATCTTAAATTTGCCGCTTTA
Ler-0_VTC1_GenBank_gDNA       GATTTTGCTAATAAACCCATGATCCTTCATCAGGTAATCTATCTTAAATTTGCCGCTTTA
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                              3550      3560      3570      3580      3590      3600
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     GTCTGCCAGTTCTTACCTATGCCTATGTTTGAACCGAGGCATGTTTTCTTGTAGATAGAG
vtc1-1_VTC1_gDNA         GTCTGCCAGTTCTTACCTATGCCTATGTTTGAACCGAGGCATGTTTTCTTGTAGATAGAG
svt2_VTC1_gDNA           GTCTGCCAGTTCTTACCTATGCCTATGTTTGAACCGAGGCATGTTTTCTTGTAGATAGAG
Ler-0_VTC1_GenBank_gDNA  GTCTGCCAGTTCTTACCTATGCCTATGTTTGAACCGAGGCATGTTTTCTTGTAGATAGAG

                              3610      3620      3630      3640      3650      3660
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     GCTCTTAAGGCAGTTGGAGTTGATGAAGTGGTTTTGGCCATCAATTATCAGCCAGAGGTA
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     AGATACTAATCTCTCTTAACTTTTTTTTTTGCAGCTATTTTCTGTTTACATATGTTTGTA
vtc1-1_VTC1_gDNA         AGATACTAATCTCTCTTAACTTTTTTTTTTGCAGCTATTTTCTGTTTACATATGTTTGTA
svt2_VTC1_gDNA           AGATACTAATCTCTCTTAACTTTTTTTTTTGCAGCTATTTTCTGTTTACATATGTTTGTA
Ler-0_VTC1_GenBank_gDNA  AGATACTAATCTCTCTTAACTTTTTTTTTTGCAGCTATTTTCTGTTTACATATGTTTGTA

                              3730      3740      3750      3760      3770      3780
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     TTTACCATTTGCTCTGTTTCGACAGGTGATGCTGAACTTCTTGAAGGACTTTGAGACCAA
vtc1-1_VTC1_gDNA         TTTACCATTTGCTCTGTTTCGACAGGTGATGCTGAACTTCTTGAAGGACTTTGAGACCAA
svt2_VTC1_gDNA           TTTACCATTTGCTCTGTTTCGACAGGTGATGCTGAACTTCTTGAAGGACTTTGAAACCAA
Ler-0_VTC1_GenBank_gDNA  TTTACCATTTGCTCTGTTTCGACAGGTGATGCTGAACTTCTTGAAGGACTTTGAGACCAA

                              3790      3800      3810      3820      3830      3840
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     GCTGGAAATCAAAATCACTTGCTCACAAGAGACCGAGCCACTAGGTACCGCTGGTCCTCT
vtc1-1_VTC1_gDNA         GCTGGAAATCAAAATCACTTGCTCACAAGAGACCGAGCCACTAGGTACCGCTGGTCCTCT
svt2_VTC1_gDNA           GCTGGAAATCAAAATCACTTGCTCACAAAAAACCGAGCCACTAGGTACCGCTGGTCCTCT
Ler-0_VTC1_GenBank_gDNA  GCTGGAAATCAAAATCACTTGCTCACAAGAGACCGAGCCACTAGGTACCGCTGGTCCTCT

                              3850      3860      3870      3880      3890      3900
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     GGCTCTAGCGAGAG~~~~~~~~~~~~~~~~~~~~~~~~~~ACAAATTGCTTGATGGATCT
vtc1-1_VTC1_gDNA         GGCTCTAGCGAGAG~~~~~~~~~~~~~~~~~~~~~~~~~~ACAAATTGCTTGATGGATCT
svt2_VTC1_gDNA           GGCTCTAGCGAGAG~~~~~~~~~~~~~~~~~~~~~~~~~~ACAAATTGCTTGATGGATCT
Ler-0_VTC1_GenBank_gDNA  GGCTCTAGCGAGAGGTTGGCGTAATCATGGTCATAGCTAGACAAATTGCTTGATGGATCT

                              3910      3920      3930      3940      3950      3960
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     GGAGAGCCCTTCTTTGTTCTTAACAGTGATGTGATTAGTGAGTACCCTCTTAAAGAAATG
vtc1-1_VTC1_gDNA         GGAGAGCCCTTCTTTGTTCTTAACAGTGATGTGATTAGTGAGTACCCTCTTAAAGAAATG
svt2_VTC1_gDNA           GGAAAGCCCTTCTTTGTTCTTAACAGTGATGTGATTAGTGAGTACCCTCTTAAAGAAATG
Ler-0_VTC1_GenBank_gDNA  GGAGAGCCCTTCTTTGTTCTTAACAGTGATGTGATTAGTGAGTACCCTCTTAAAGAAATC

                              3970      3980      3990      4000      4010      4020
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     CTTGAGTTTCACAAATCTCACGGTGGGGAAGCCTCCATAATGGTAACAAAGGTGAGATTA
vtc1-1_VTC1_gDNA         CTTGAGTTTCACAAATCTCACGGTGGGGAAGCCTCCATAATGGTAACAAAGGTGAGATTA
svt2_VTC1_gDNA           CTTGAGTTTCACAAATCTCACGGTGGGGAAGCCTCCATAATGGTAACAAAGGTGAGATTA
Ler-0_VTC1_GenBank_gDNA  CTTGAGTTTCACAAATCTCACGGTGGGGAAGCCTCCATAATGGTAACAAAGGTG~GATTA

                              4030      4040      4050      4060      4070      4080
                         ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA     TCGAAACATAATACTCTCCAGTTACGAGATAAGTACGTTATTCATCTAATGTGGACTTGC
```

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vtc1-1_VTC1_gDNA          TCGAAACATAATACTCTCCAGTTACGAGATAAGTACGTTATTCATCTAATGTGGACTTGC
svt2_VTC1_gDNA            TCGAAACATAATACTCTCCAGTTACGAGATAAGTACGTTATTCATCTAATGTGGACTTGC
Ler-0_VTC1_GenBank_gDNA   TCGAAACATAATACTCTCCAGTTACGAGATAAGTACGTTATTCATCTAATCTGGACTTGC

                                   4090      4100      4110      4120      4130      4140
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      ATGTATTGGTTATATAGGTGGATGAACCGTCGAAATATGGAGTGGTTGTTATGGAAGAAA
vtc1-1_VTC1_gDNA          ATGTATTGGTTATATAGGTGGATGAACCGTCGAAATATGGAGTGGTTGTTATGGAAGAAA
svt2_VTC1_gDNA            ATGTATTGGTTATATAGGTGGATGAACCGTCGAAATATGGAGTGGTTGTTATGGAAGAAA
Ler-0_VTC1_GenBank_gDNA   ATGTATTGGTTATATAGGTGGATGAACCGTCGAAATATGGAGTGGTTGTTATGGAAGAAA

                                   4150      4160      4170      4180      4190      4200
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      GCACTGGAAGAGTGGAGAAGTTTGTGGAAAAGCCAAAACTGTATGTAGGTAACAAGATCA
vtc1-1_VTC1_gDNA          GCACTGGAAGAGTGGAGAAGTTTGTGGAAAAGCCAAAACTGTATGTAGGTAACAAGATCA
svt2_VTC1_gDNA            GCACTGGAAGAGTGGAGAAGTTTGTGGAAAAGCCAAAACTGTATGTAGGTAACAAGATCA
Ler-0_VTC1_GenBank_gDNA   GCACTGGAAGAGTGGAGAAGTTTGTGGAAAAGCCAAAACTGTATGTAGGTAACAAGATCA

                                   4210      4220      4230      4240      4250      4260
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      ACGCTGGGATTTATCTTCTGAACCCATCTGTTCTTGATAAGATTGAGCTAAGACCGACTT
vtc1-1_VTC1_gDNA          ACGCTGGGATTTATCTTCTGAACCCATCTGTTCTTGATAAGATTGAGCTAAGACCGACTT
svt2_VTC1_gDNA            ACGCTGGGATTTATCTTCTGAACCCATCTGTTCTTGATAAGATTGAGCTAAGACCGACTT
Ler-0_VTC1_GenBank_gDNA   ACGCTGGGATTTATCTTCTGAACCCATCTGTTCTTGATAAGATTGAGCTAAGACCGACTT

                                   4270      4280      4290      4300 ↓    4310      4320
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      CAATCGAAAAAGAGACTTTCCCTAAGATTGCAGCAGCGCAAGGGCTCTATGCTATGGTGC
vtc1-1_VTC1_gDNA          CAATCGAAAAAGAGACTTTCCCTAAGATTGCAGCAGCGCAAGGGCTCTATGCTATGGTGC
svt2_VTC1_gDNA            CAATCGAAAAAGAGACTTTCCCTAAGATTGCAGCAGCGCAAGGGCTCTATGCTATGGTGC
Ler-0_VTC1_GenBank_gDNA   CAATCGAAAAAGAGACTTTCCCTAAGATTGCAGCAGCGCAAGGGCACTATGCTATGGTGC

                                   4330      4340      4350      4360      4370      4380
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      TACCAGGGTTTTGGATGGACATTGGGCAACCCCGTGACTACATAACGGGTTTGAGACTCT
vtc1-1_VTC1_gDNA          TACCAGGGTTTTGGATGGACATTGGGCAACCCCGTGACTACATAACGGGTTTGAGACTCT
svt2_VTC1_gDNA            TACCAGGGTTTTGGATGGACATTGGGCAACCCCGTGACTACATAACGGGTTTGAGACTCT
Ler-0_VTC1_GenBank_gDNA   TACCAGGGTTTTGGATGGACATTGGGCAACCCCGTGACTACATAACGGGTTTGAGACTCT

                                   4390      4400      4410      4420      4430      4440
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      ACTTAGACTCCCTTAGGAAGAAATCTCCTGCCAAATTAACCAGTGGGCCACACATAGTTG
vtc1-1_VTC1_gDNA          ACTTAGACTCCCTTAGGAAGAAATCTCCTGCCAAATTAACCAGTGGGCCACACATAGTTG
svt2_VTC1_gDNA            ACTTAGACTCCCTTAGGAAGAAATCTCCTGCCAAATTAACCAGTGGGCCACACATAGTTG
Ler-0_VTC1_GenBank_gDNA   ACTTAGACTCCCTTAGGAAGAAATCTCCTGCCAAATTAACCAGTGGGCCACACATAGTTG

                                   4450      4460      4470      4480      4490      4500
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      GGAATGTTCTTGTTGACGAAACCGCTACAATTGGGGAAGGATGTTTGATTGGACCAGACG
vtc1-1_VTC1_gDNA          GGAATGTTCTTGTTGACGAAACCGCTACAATTGGGGAAGGATGTTTGATTGGACCAGACG
svt2_VTC1_gDNA            GGAATGTTCTTGTTGACGAAACCGCTACAATTGGGGAAGGATGTTTGATTGGACCAGACG
Ler-0_VTC1_GenBank_gDNA   GGAATGTTCTTGTTGACGAAACCGCTACAATTGGGGAAGGATGTTTGATTGGACCAGACG

                                   4510      4520      4530      4540      4550      4560
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      TTGCCATTGGTCCAGGCTGCATTGTTGAGTCAGGAGTCAGACTCTCCCGATGCACGGTCA
vtc1-1_VTC1_gDNA          TTGCCATTGGTCCAGGCTGCATTGTTGAGTCAGGAGTCAGACTCTCCCGATGCACGGTCA
svt2_VTC1_gDNA            TTGCCATTGGTCCAGGCTGCATTGTTGAGTCAGGAGTCAGACTCTCCCGATGCACGGTCA
Ler-0_VTC1_GenBank_gDNA   TTGCCATTGGTCCAGGCTGCATTGTTGAGTCAGGAGTCAGACTCTCCCGATGCACGGTCA
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                                     4570      4580      4590      4600      4610      4620
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        TGCGTGGAGTCCGCATCAAGAAGCATGCGTGTATCTCGAGCAGTATCATCGGGTGGCACT
vtc1-1_VTC1_gDNA            TGCGTGGAGTCCGCATCAAGAAGCATGCGTGTATCTCGAGCAGTATCATCGGGTGGCACT
svt2_VTC1_gDNA              TGCGTGGAGTCCGCATCAAGAAGCATGCGTGTATCTCGAGCAGTATCATCGGGTGGCACT
Ler-0_VTC1_GenBank_gDNA     TGCGTGGAGTCCGCATCAAGAAGCATGCGTGTATCTCGAGCAGTATCATCGGGTGGCACT

                                     4630      4640      4650      4660      4670      4680
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        CAACGGTTGGTCAATGGGCCAGGATCGAGAACATGACGATCCTCGGTGAGGATGTTCATG
vtc1-1_VTC1_gDNA            CAACGGTTGGTCAATGGGCCAGGATCGAGAACATGACGATCCTCGGTGAGGATGTTCATG
svt2_VTC1_gDNA              CAACGGTTGGTCAATGGGCCAGGATCGAGAACATGACGATCCTCGGTGAGGATGTTCATG
Ler-0_VTC1_GenBank_gDNA     CAACGGTTGGTCAATGGGCCAGGATCGAGAACATGACGATCCTCGGTGAGGATGTTCATG

                                     4690      4700      4710      4720      4730      4740
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        TGAGCGATGAGATCTATAGCAATGGAGGAGTTGTTTTGCCACACAAGGAGATCAAATCAA
vtc1-1_VTC1_gDNA            TGAGCGATGAGATCTATAGCAATGGAGGAGTTGTTTTGCCACACAAGGAGATCAAATCAA
svt2_VTC1_gDNA              TGAGCGATGAGATCTATAGCAATGGAGGAGTTGTTTTGCCACACAAGGAGATCAAATCAA
Ler-0_VTC1_GenBank_gDNA     TGAGCGATGAGATCTATAGCAATGGAGGAGTTGTTTTGCCACACAAGGAGATCAAATCAA

                                     4750      4760      4770      4780  ↓↓  4790      4800
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        ACATCTTGAAGCCAGAGATAGTGATGTGAAAATGAGATATTATA~~TGTGCAACTTTTTT
vtc1-1_VTC1_gDNA            ACATCTTGAAGCCAGAGATAGTGATGTGAAAATGAGATATTATA~~TGTGCAACTTTTTT
svt2_VTC1_gDNA              ACATCTTGAAGCCAGAGATAGTGATGTGAAAATGAGATATTATA~~TGTGCAACTTTTTT
Ler-0_VTC1_GenBank_gDNA     ACATCTTGAAGCCAGAGATAGTGATGTGAAAATGAGATATTATATATGTGCAACTTTTTT

                                     4810      4820      4830      4840      4850      4860
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        TTTTTTTTTT~GTGTCCTTTCTTCAACTTTGAAATCGCTTTCGTAATTCTTAATGGCTTT
vtc1-1_VTC1_gDNA            TTTTTTTTTT~GTGTCCTTTCTTCAACTTTGAAATCGCTTTCGTAATTCTTAATGGCTTT
svt2_VTC1_gDNA              TTTTTTTTTT
Ler-0_VTC1_GenBank_gDNA     TTTTTTTTTTTGTGTCCTTTCTTCAACTTTGAAATCGCTTTCGTAATTCTTAATGGCTTT

                                     4870      4880      4890      4900      4910      4920
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        TGAATAAGCATCAATCAAAACGCTGTATATCTTGTTAGGGTCGTTTGCTGTTTTGTCTCT
vtc1-1_VTC1_gDNA            TGAATAAGCATCAATCAAAACGCTGTATATCTTGTTAGGGTCGTTTGCTGTTTTGTCTCT
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA     TGAATAATCATCAATCAAAACGCTGTATATCTTGTTAGGGTCGTTTGCTGTTTTGTCTCT

                                     4930      4940      4950      4960      4970      4980
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        TTTTTTGTTTTGTAATTTATAAAAAAATTTATTCTCATTTTATGTGAGATACTTTTGAAT
vtc1-1_VTC1_gDNA            TTTTTTGTTTTGTAATTTATAAAAAAATTTATTCTCATTTTATGTGAGATACTTTTGAAT
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA     TTTTTTGTTTTGTAATTTATAAAAAAAATTATTCTCATTTTATGTGAGATACTTTTGAAT

                                     4990      5000      5010      5020      5030      5040
                            ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA        ATTCATTAATTATAAAGCTTTTTTTTTGTGAAGTAACATTCAAAATTCAATAGTAGTCAT
vtc1-1_VTC1_gDNA            ATTCATTAATTATAAAGCTTTTTTTTTGTGAAGTAACATTCAAAATTCAATAGTAGTCAT
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA     ATTCATTAATTATAAAGCTTTTTTTTTGTGAAGTAACATTCAAAATTGAATAGTAGTCAT

                                     5050      5060      5070      5080      5090      5100
                            ....|....|....|....|....|....|....|....|....|....|....|....|
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Col-0_VTC1_TAIR_gDNA      TGTAAAAAAACTTGAAAATAGCATGTATAATGCAGATAAAATTTTTTAACATGACCATTG
vtc1-1_VTC1_gDNA          TGTAAAAAAACTTGAAAATAGCATGTATAATGCAGATAAAATTTTTTAACATGACCATTG
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA   TATAAAAAAACTTGAAAATAGAATGTATAATGCAGATAAAATTTTTTAACATGACCATTG

                                   5110      5120      5130      5140      5150      5160
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      ATAATCCAAAAAGGTTTTAAAAATTAGAAGCAACAAATATCCATTGGGGTTTCTCCAAAT
vtc1-1_VTC1_gDNA          ATAATCCAAAAAGGTTTTAAAAATTAGAAGCAACAAATATCCATTGGGGTTTCTCCAAAT
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA   ATAATCCAAAAAGGTTTTAAAAATTAGAAGCAACAAATATCCATTGGGGTTTCTCCAAAT

                                   5170      5180      5190      5200      5210      5220
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      CCAAGGCATAGAGGAATATTCGTTAGCGTGGAAAGACTAAAATACCCTTAACTTCCCTCC
vtc1-1_VTC1_gDNA          CCAAGGCATAGAGGAATATTCGTTAGCGTGGAAAGACTAAAATACCCTTAACTTCCCTCC
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA   CCAAGGCATAGAGGAATATTCGTTAGCGTGGAAAGACTAAAATACCCTTAACTTCCCTCC

                                   5230      5240      5250      5260      5270      5280
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA      ACATAATGTATACAGTGACCCGATCTGAATTC
vtc1-1_VTC1_gDNA          ACATAATGTATACAGTGACCCGATCTGAATTC
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA   ACATAATGTATACAGTGACCCGATCTGAATTCGGAGCTAAAAACGGTTTCAATCGAATTA

                                   5290      5300      5310      5320      5330      5340
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA
vtc1-1_VTC1_gDNA
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA   AAGTCAATGGCGTCACGTTTATGTCTTCTCCTTCTCGTTGCGTGTATCGCCGGAGCATTT

                                   5350      5360      5370      5380      5390      5400
                          ....|....|....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_TAIR_gDNA
vtc1-1_VTC1_gDNA
svt2_VTC1_gDNA
Ler-0_VTC1_GenBank_gDNA   GCCGGAGACGTCATCGAACTCAATCGATCTCAGAGGGAGTTCGATTATTTCGCTCTATCT
```

Figure 2.6 Sequence alignment of the *VTC1* gene sequence of the Col-0 TAIR database, the *vtc1-1*, *svt2* mutants, and the L*er*-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 1902 and 2273). Upright arrows indicate sequences shared between *svt2* and L*er*. Arrows pointing down denote sequences shared between *svt2* and Col. Arrow heads point to sequences unique to *svt2*. Highlighted in yellow are the start and stop codons, respectively. Highlighted in green is the *vtc1-1* mutation.

Table 2.3 Summary of *svt2* revertants 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 L*er*-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 2).

Generation	**Total # of plants**	**# of phenotypic revertants**	**% reversion**
svt2 M_1 (experiment 1)	63	0	0
svt2 M_1 (experiment 2)	96	1 (Col phenotype)	1.04
svt2 M_2 (experiment 1), 3 of 7 revertants tested further:	78	7 (Col phenotype)	8.97
↳ *svt2* A8 Col R M_3	64	0	0
↳ *svt2* G7 Col R M_3	64	0	0
↳ *svt2* K1 Col R M_3*	63	1 (*svt2* phenotype)	1.58
↳ *svt2* K1 Col R *svt2* R M_4*	96	0	0
svt2 M_2 (experiment 2), 2 of 5 revertants tested further:	62	5 (Col phenotype)	8.06
↳ *svt2* Col R1 M_3*	88	20 (*svt2* phenotype)	22.73
↳ *svt2* Col R4 M_3*	96	0	0
svt2 M_2 (experiment 3)	96	10 (Col phenotype)	10.42
svt2 M_3 (experiment 1)	96	0	0

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. Fig. 2.7) illustrates the phenotypic appearance of three examples of *svt2* $\rightarrow$ Col single revertants (Col R1, Col R2, K1 Col R) and an *svt2* $\rightarrow$ Col $\rightarrow$ *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* $\rightarrow$ Col $\rightarrow$ *svt2* double revertant phenotype corresponds with *svt2*-like genomic markers. To check this we isolated genomic DNA from Col-0 and L*er*-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. In all cases but the *svt2* M_2 Col R1 revertant, the *svt2*-like revertant plants (labeled *svt2* M_2 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* M_1 plants and *svt2* M_2 plants that displayed a *svt2* phenotype, gave rise to PCR products that were of the same electrophoretic mobility as those of the L*er* wild type (Table 2.4, Fig. 2.8). In addition, the double revertant plant K1 (labeled *svt2* M_2 K1 Col R) was genotyped in both its M_2 and M_3 generations. The K1 plant produced InDel PCR products similar to those of the Col-0 wild type in the M_2 generation. However, the M_3 generation that displayed *svt2*-like morphology produced PCR products that were comparable to the InDel PCR products generated using L*er* genomic DNA (Table 2.4). The *svt2* M_2 Col R1 (highlighted in red in Table 2.4) is intriguing, because it appears to contain DNA that is similar to both Col and L*er* genomic DNA. This suggests the presence of chimeric genome sectors, which may explain the higher percentage of Col-like revertants compared to *svt2* M_2 K1 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 L*er* wild-type, respectively (Fig. 2.9).

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.

2.3.5 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

Table 2.4 Summary of PCR-based molecular genotypes in Col-0, *vtc1-1*, L*er*-0, *svt2*, and revertants. With the exception of *svt2* Col R1 M_2, where Col and L*er* 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 L*er*-like phenotype correlated with L*er* polymorphisms. C, L, and H refer to Col, L*er*, or heterozygous, respectively. R denotes revertant.

Genotype	InDel 1 450919	InDel 2 451470	InDel 3 469762	InDel 4 449053	InDel 5 455100	G1F + G2R *VTC1*
Col-0 WT	C	C	C	C	C	C
vtc1-1	C	C	C	C	C	C
L*er*-0 WT	L	L	L	L	L	L
svt2 M_1	L	L	L	L	L	L
svt2 M_2	L	L	L	L	L	L
svt2 Col R1 M_2	C	L	C	H	C	C
svt2 Col R2 M_2	C	C	C	C	C	C
svt2 Col R3 M_2	C	C	C	C	C	C
svt2 Col R4 M_2	C	C	C	C	C	C
svt2 Col R5 M_2	C	C	C	C	C	C
svt2 K1 Col R M_2	C	C	C	C	C	C
svt2 K1 Col R *svt2* R M_3	L	L	L	L	L	L

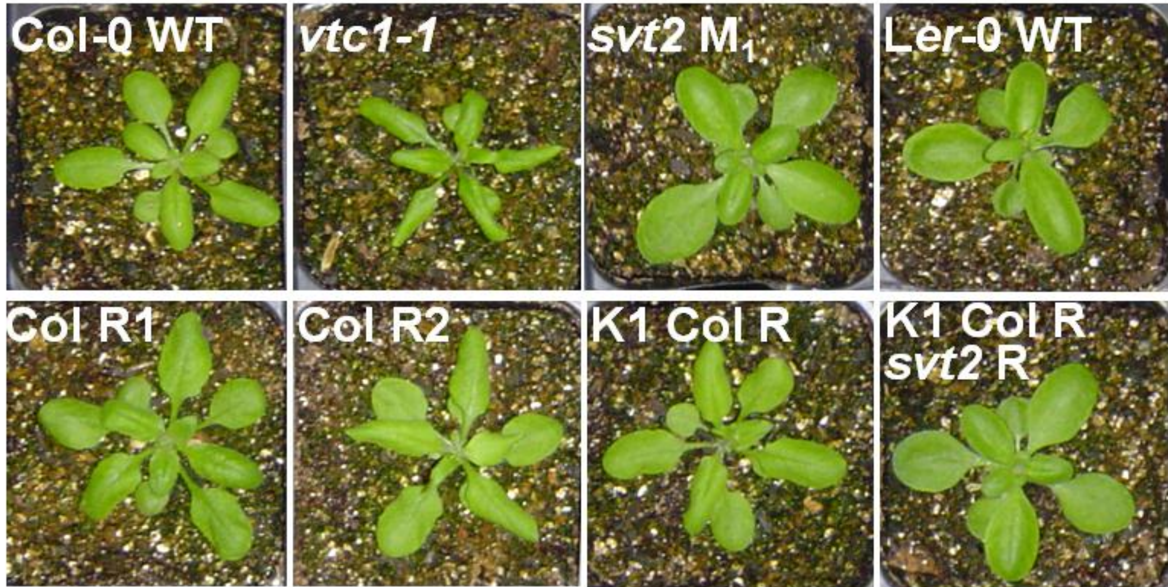

Figure 2.7 Photographs showing the phenotype of *svt2* revertants.

Plants were three weeks old when photographs were taken. Top row represents controls, Col-0 wild type, *vtc1-1* and *svt2* mutants, and L*er*-0 wild type. Bottom row represents three Col-like revertants, *svt2* Col R1 M_3, *svt2* Col R2 M_3, *svt2* K1 Col R M_3, and a double revertant, *svt2* K1 Col R *svt2* R M_4. R stands for revertant.

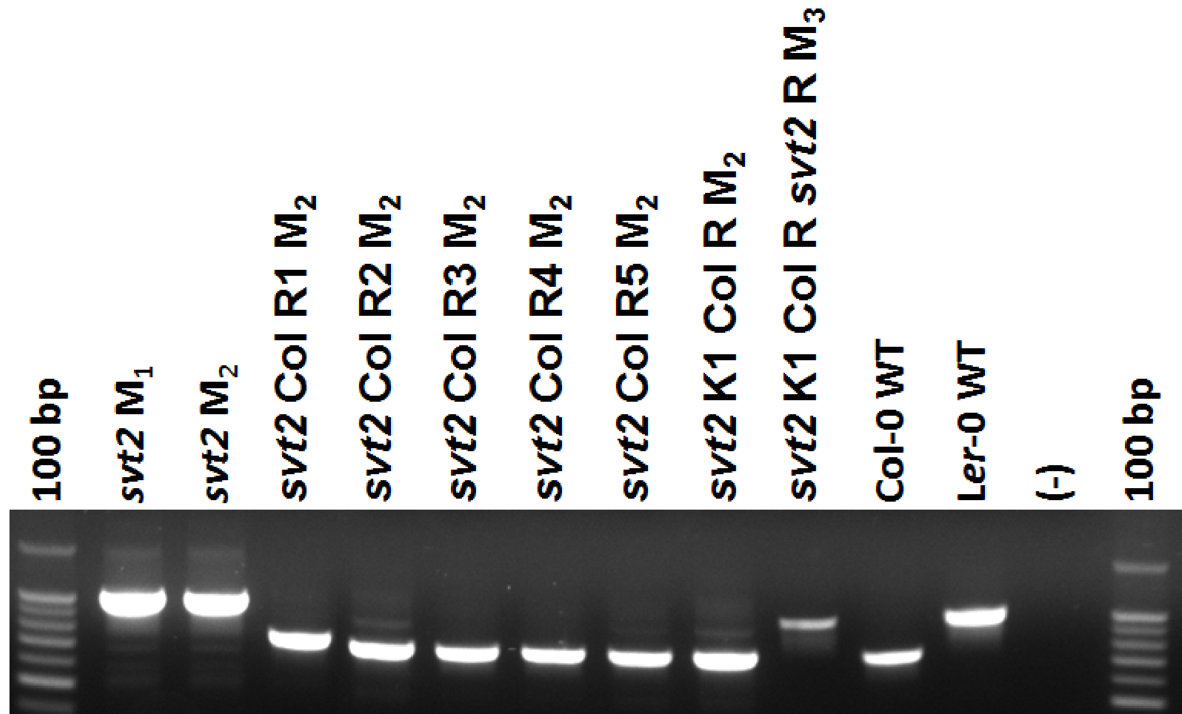

Figure 2.8 Gel imagine showing InDel polymorphisms in *svt2*, Col-0, L*er*-0, and revertants.
PCR amplification of the Col/Ler *VTC1* promoter polymorphism in *svt2* plants and *svt2* revertant (R) plants, amplified with the *VTC1* G1F and G2R primers. (-) indicates negative control, no DNA.

```
                                1760      1770      1780      1790      1800
                            ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR        GATTTACTTACATTTAACTAATTTTTAAGGTCTGTTGATCGAACTCGTTA
Ler-0_VTC1_gDNA_Genbank     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R M3_G1F        --------------------------------------------------
svt2 K1 Col R svt2 R M4_G1F ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                1810      1820      1830      1840      1850
                            ....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR        CCTTGAACAATTAGTAGGACAACTCAAACCACTAAACCATTATACTTTAA
Ler-0_VTC1_gDNA_Genbank     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R M3_G1F        --------------------------------------------------
svt2 K1 Col R svt2 R M4_G1F ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                1860      1870      1880      1890      1900
                            ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR        GGATTTATGTAAAATTTCAAATATATATAGTTTAGTAGATGCACTTATCA
Ler-0_VTC1_gDNA_Genbank     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R M3_G1F        --------------------------------------------------
svt2 K1 Col R svt2 R M4_G1F ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                1910      1920      1930      1940      1950
                            ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR        TCACACTCACCAATTGGATGTCAACACCTGGTTCTAGCTTTTTAATTACC
Ler-0_VTC1_gDNA_Genbank     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R M3_G1F        --------------------------------------------------
svt2 K1 Col R svt2 R M4_G1F ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                1960      1970      1980      1990      2000
                            ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR        AAAGTGAAAAAACTGACTTTTTCTAAAAAAAAATTCGTTCTAGATGGATGC
Ler-0_VTC1_gDNA_Genbank     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TCGNATCTAGATGGATGC
svt2 K1 Col R M3_G1F        --------------------------~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R svt2 R M4_G1F ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                             <------
                                2010      2020      2030      2040      2050
                            ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR        TCTTCAAATTCGTTCTAAAAAAAACTGA~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_gDNA_Genbank     TCTTTAAAT~CTTCGATATTTTTTATCCGTTTCGATAATATGGTAAGAAT
svt2 K1 Col R M3_G1F        ~~~~~~~~ATAGTCGCTCTAGAATGTGA~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R svt2 R M4_G1F ~~~~ATCAGCTTCTAGCATGGATGCTCCGTTTCGATAATAATGTGAGAAT

                                2060      2070      2080      2090      2100
                            ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_gDNA_Genbank     GAATGACGAATCGGTCAAGCTAATCTGTATATTAA CATTGTACTCATCA
svt2 K1 Col R M3_G1F        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R svt2 R M4_G1F GAATGACGAATCGGTCAAGCTAATCTGTATATTAATCATTGTACTCATCA

                                2110      2120      2130      2140      2150
```

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                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_gDNA_Genbank       ACGTAAAGTCCTATTCGTCTATACATATGTGAACTTATATATGTCTATCA
svt2 K1 Col R M3_G1F          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R svt2 R M4_G1F   ACGTAAAGTCCTATTCGTCTATACATATGTGAACTTATATATGTCTATCA

                                       2160      2170      2180      2190      2200
                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_gDNA_Genbank       ACTAGTT  CACTACCCTATAAAGTTCATCAGAGTTGTCGATCAGCAGTG
svt2 K1 Col R M3_G1F          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R svt2 R M4_G1F   ACTAGTTCACACTACCCTATAAAGTTCATCAGAGTTGTCGATCAGCAGTG
                                       2210      2220      2230      2240      2250
                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Ler-0_VTC1_gDNA_Genbank       ACCACTACACATTCTTCATACAGCTGAGTTAGGAATGTTAACAAAATAGT
svt2 K1 Col R M3_G1F          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
svt2 K1 Col R svt2 R M4_G1F   ACCACTACACATTCTTCATACAGCTGAGTTAGGAATGTTAACAAAATAGT

                                       2260      2270      2280      2290      2300
                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          ~~~~~~~~~~~~~~~~~~~ACATAATACCATCCCTTTAAAAACACAGAAT
Ler-0_VTC1_gDNA_Genbank       TTATGGGAGTATGTTTTATACATAATACCATCCCTTTAAAAACACAGAAT
svt2 K1 Col R M3_G1F          ~~~~~~~~~~~~~~~~~~~~CATAATACCATCCCTTTAAAA-CACAGAAT
svt2 K1 Col R svt2 R M4_G1F   TTATGGGAGTATGTTTTATACATAATACCATCCCTTTAAAAACACAGAAT

                                       2310      2320      2330      2340      2350
                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          TTTTTTTATCATCTCTGAAACAAATCATTTACAGTAGTAAATGTCAA--C
Ler-0_VTC1_gDNA_Genbank       TTTCTTTATCATCTCCGAAACAAATCATTTACAGTAGTAAATGTAAAAAC
svt2 K1 Col R M3_G1F          TTTTTTTATCATCTCTGAAACAAATCATTTACAGTAGTAAATGTCAA--C
svt2 K1 Col R svt2 R M4_G1F   TTTCTTTATCATCTCCGAAACAAATCATTTACAGTAGTAAATGTAAAAAC

                                       2360      2370      2380      2390      2400
                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          ACAACATTAATTCTGTTT-GTTGTTGGCATTTACAATTGCAAAATCATTT
Ler-0_VTC1_gDNA_Genbank       ACAACATTAATTCTGTTT-GTTGTTGGCATTTACAATTGCAAAATCATTT
svt2 K1 Col R M3_G1F          ACAACATTAATTTGGTTTTGTTGTTGGCATTTACAATTGCAAAATCATTT
svt2 K1 Col R svt2 R M4_G1F   ACAACATTAATTCTGTTT-GTTGTTGGCATTTACAATTGCAAAATCATTT

                                       2410      2420      2430      2440      2450
                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          TCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAAT
Ler-0_VTC1_gDNA_Genbank       TCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAAT
svt2 K1 Col R M3_G1F          TCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAAT
svt2 K1 Col R svt2 R M4_G1F   TCTCATTTATTATTCGTATTTATTTTGTCAAGAACCCTTGTCTCTAAAAT

                                       2460      2470      2480      2490      2500
                              ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR          ATTCATAGAAAAAGAAAAGAGCCATTAATTAATGGCTTGAAGAAAGA-TT
Ler-0_VTC1_gDNA_Genbank       ATTCATAGAAAAA-AAAAGAGCCATTAATTAATGGCTTGAAGAAAGA-TT
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svt2 K1 Col R M3_G1F         ATTCATAGAAAAAGAAAAGAGCCATTAATTAATGGCTTGAAGAAAGA-TT
svt2 K1 Col R svt2 R M4_G1F  ATTCATAGAAAAA-AAAAGAGCCATTAATTAATGGCTTGAAGAAAGAATT

                                       2510      2520      2530      2540      2550
                             ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR         GGTGTATAAGC-GTCTACGTGACCTTTAA--TTAATTTACTT---CCCCC
Ler-0_VTC1_gDNA_Genbank      GGTGTATAAGC-GTCTACGTGACCTTTAA--TTAATTTACTT---CCCCA
svt2 K1 Col R M3_G1F         GGTGTATAAGC-GTCTACGTGACCTTTAA--TTAATTTACTT---CCCCC
svt2 K1 Col R svt2 R M4_G1F  GGTGTATAAACCGTCTACCTGACCTTTAAATTTAATTTACTTTCCCCCCA
                                       2560      2570      2580      2590      2600
                             ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR         AAAAAAGTCAAC-ATTCAA-CATGTG-AATAAAAA-TCAATATTGG-TTT
Ler-0_VTC1_gDNA_Genbank      AAAAAAGTCAAC-ATTCAA-CATGTG-AATAAAAAATCAATATTGG-TTT
svt2 K1 Col R M3_G1F         AAAAAAGTCAAC-ATTCAA-CATGTG-AATAAAAA-TCAATATTGG-TTT
svt2 K1 Col R svt2 R M4_G1F  CAAAAAGTCAACCATTCAAACATGGGGAGTAAAAAATCAATATTGGGTTG

                                       2610      2620      2630      2640      2650
                             ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR         CTAAGTAA-GTAAGTACCATATTATTAAATTATTTATTTT-GGTAAATAC
Ler-0_VTC1_gDNA_Genbank      CTAAGTAA-GTAAGTACCATATTATTAAATTATTT-ATTTTGGTAAATAC
svt2 K1 Col R M3_G1F         CTAAGTAA-GTAAGTACCATATTATTAAATTATTTATTTT-GGTAAATAC
svt2 K1 Col R svt2 R M4_G1F  CTAAGTAAAGTAAGTACCATATTATTAAATTATTTTATTTTGGTAAAAGA
                                       2660      2670      2680      2690      2700
                             ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR         --GCACTCAATTTTTCTC--TCAACG----GTGTATATAAACAAAAGGAG
Ler-0_VTC1_gDNA_Genbank      --GCACTCAATTTTTCTC--TCAACGGTGGGTGTATATAAACAAAAGGAG
svt2 K1 Col R M3_G1F         --GCACTCAATTTTTCTC--TCAACG----GTGTATATAAACAAAAGGAG
svt2 K1 Col R svt2 R M4_G1F  CAACACTCAATTTTTTCTCTTCACCGGGGGGGGATGTAAAAAAAAAGAG

                                       2710      2720      2730      2740      2750
                             ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR         TCTCCTTTGGAAAAAACTTGCCTATCATTTTGCCAACGAAC-GTTCTTTC
Ler-0_VTC1_gDNA_Genbank      TCTCCTTTGGAAAAAACTTGCCTATCATTTTGCCAACGAAC-GTTCTTTC
svt2 K1 Col R M3_G1F         TCTCCTTTGGAAAAAACTTGCCTATCATTTTGCCAACGAAC-GTTCTTTC
svt2 K1 Col R svt2 R M4_G1F  TCTCCTTTGGAAAAAAAAAAC-TATC-TTTTGCCGCC-AAC-ATTTTTTT

                                       2760      2770      2780      2790      2800
                             ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR         TTCTTAATCAC-AGCTCAGCCTGACGCAACCGCTCAGGCTGATCTCTTCC
Ler-0_VTC1_gDNA_Genbank      TTCTTAATCAC-AGCTCAGC-CTACGCAACCGCTCAGGCTGATCTCTTCC
svt2 K1 Col R M3_G1F         TTCTTAATCAC-AGCTCAGCCTGACGCAACCGCTCAGGCTGATTGATTCT
svt2 K1 Col R svt2 R M4_G1F  TCTTTATTCA---GCTCAGC-CTGAGGAACCCCCAGTGGGCATTTTTTAA

                                       2810      2820      2830      2840      2850
                             ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR         AATTTACAGCCATTTCCCAGCTCAGATCTCTGATCCGGTGAGATCTCTCT
Ler-0_VTC1_gDNA_Genbank      AATTTACAGCCATTTCCCAGCTCAGATCTCTGATCCGGTGAGATCTCTCT
svt2 K1 Col R M3_G1F         CTTTTTT--TCCCCGAAAAAAAAAAAACAGAAACTGGGGGCAATTTTTTT
svt2 K1 Col R svt2 R M4_G1F  AAAAG---------------------------------------------

                                       2860      2870      2880      2890      2900
```

```
                                ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR            CAAGGTAATGCCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATGCAT
Ler-0_VTC1_gDNA_Genbank         CAAGGTAATGCCCCTGCAATTTTGCTTACTTCTCTGGTTGTGATATGCAT
svt2 K1 Col R M3_G1F            TA--GCACCCCTCCAAAAAGGAAATTTTTTAATCTTCTGTCCAAAACCGT
svt2 K1 Col R svt2 R M4_G1F     --------------------------------------------------

                                        2910      2920      2930      2940      2950
                                ....|....|....|....|....|....|....|....|....|....|
Col-0_VTC1_gDNA_TAIR            GTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCTCATTTTGTATTTGC
Ler-0_VTC1_gDNA_Genbank         GTTCTTCGAATTTTCATCGTTTGTGATTTGAATTCTCATTTTGTATTT-C
svt2 K1 Col R M3_G1F            ATTTTTTTAAGCCCCCCTTT------------------------------
svt2 K1 Col R svt2 R M4_G1F     --------------------------------------------------
svt2 K1 Col R svt2 R M4_G1R     --------------------------------------------------
```

Figure 2.9 Sequence alignment of the *VTC1* promoter InDel polymorphism sequence of the Col-0 TAIR database, the L*er*-0 Genbank database, the *svt2* K1 Col R M_3 (Col-like phenotype) and the *svt2* K1 Col R *svt2* R M_4 (*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 L*er*-0 and *svt2* K1 Col R *svt2* R M_4, which exhibits an *svt2*-like phenotype. The *svt2* K1 Col R M_3 mutant has a Col-like phenotype and share sequence similarities with the Col-0 sequence. R denotes revertant.

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 from one generation to the next, termed transgenerational epigenetic inheritance (Molinier et al., 2006; Jablonka and Raz, 2009). 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 an *svt2*-like phenotype. Generally, F_1 plants were much larger than *svt2*, but appeared to have a "mixed" Col/*svt2* phenotype (data not shown). To determine the genotypes of the F_1 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 2.5 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 L*er* genomic DNA, respectively, were generated, suggesting that the F_1 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 L*er*-0 wild-type DNA were prevalent (highlighted in red in Table 2.5). 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.

2.4 DISCUSSION

Although this study aimed to isolate novel suppressor mutants of the AA-deficient *Arabidopsis* mutant *vtc1-1*, we identified *svt2*, which indeed has wild-type levels of AA (Fig. 2.2c) and recovered root development in the presence of ammonium (Fig. 2.2d). However, *svt2* manifests genotypic and phenotypic instability, making it unsuitable for the identification of VTC1 interacting partners. Instead, *svt2* harbors unique characteristics that will aid in our understanding of the mechanism of genome instability and restoration.

2.4.1 *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 that was isolated among over 1000 EMS-mutagenized M_0 seeds, showing unique phenotypic characteristics. Our genetic analysis revealed that astonishingly

Table 2.5 Summary of insertion/deletion polymorphism analysis of 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 F_1 1	H	H	L	H	H	H
svt2 x Col-0 F_1 2	H	H	L	H	H	H
svt2 x Col-0 F_1 3	H	H	H	H	H	H
svt2 x Col-0 F_1 4	H	H	H	H	H	H
Col-0 *x svt2* F_1 1	H	H	H	L	H	H
Col-0 *x svt2* F_1 2	H	H	L	H	H	H
Col-0 *x svt2* F_1 3	H	H	H	L	H	H
Col-0 *x svt2* F_1 4	H	H	L	H	H	H

both maternal and paternal alleles were affected in five randomly selected InDel polymporphism loci, the newly discovered InDel polymporphism in the *VTC1* promoter, and additional SNPs (Figs. 2.3b, c, d, 2.4, 2.5, 2.6). These data demonstrate that *svt2* has acquired new characteristics, presumably in response to EMS mutagenesis, and that *svt2* is neither Col nor L*er*. The data also argue against *svt2* being an experiment or PCR artifact.

Second, a number of data provide strong arguments against seed contamination. (1) Descendents of the original *svt2* mutant produce with high reproducibility offspring revertants with Col-like features (Tables 2.3, 2.4; Figs. 2.7, 2.8). (2) One of the Col-like revertants, *svt2* Col R1 M_3, exhibited heterozygosity at some of the InDels tested (Table 2.4). (3) One of those Col-like revertant, *svt2* K1 Col R M_3, produced progeny that reverted back to *svt2*-like plants (Tables 2.3, 2.4, Figs. 2.7, 2.8). (4) We were unable to obtain true F_1 heterozygotes in *svt2*/Col-0 reciprocal crosses (Table 2.5). (5) The delayed flowering phenotype and enlarged morphology argue against the fact that *svt2* is a result of a L*er*-0 wild-type seed landing on the flat during the initial planting of the *vtc1-1* M_0 mutagenized population. There is the possibility of a L*er* 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 L*er* seed. We performed many other experiments using this very same seed stock and never observed L*er*-like plants among the *vtc1* population. However, arguments (1) through (4) above are most compelling 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 L*er* wild-type plants. (1) If *svt2* were generated by L*er* cross-pollination, the InDel polymorphism markers tested using *svt2* genomic DNA should have indicated heterozygosity. This, however, was not the case (Table 2.4). (2) While *svt2* shares phenotypic and genotypic characteristics with L*er* and Col, it also has unique features (Figs. 2.2a, 2.6). (3) *svt2* exhibits phenotypic and genotypic instability, causing the appearance of revertants with persistent reproducibility. (4) L*er* 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.

2.4.2 Possible causes of genome instability in *svt2*

Our results are indicative of genome instability in *svt2*. Genome instability may be a result of polyploidy (Wang et al., 2010). Polyploids can arise by 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 L*er*-0 wild-type controls (Table 2.2). Furthermore, extra DNA must be replicated with each cell division. Therefore, enlarged cell size is often associated with polyploids (Ranney, 2006). The chemical mutagenesis of *vtc1-1* seed may have resulted in mutations, which may have led to increased ploidy levels in one, two, or all three meristem layers, L1, L2, L3. However, only mutations in the L2 layer, which gives rise to the reproductive organs, are inherited. Polyploidy in the L2 layer are reflected in pollen size. While *svt2* has an overall enlarged morphology (Fig. 2.2a), its pollen size is comparable to that of the other three genotypes (Fig. 2.2b). This suggests that *svt2* anthers are not polyploid. Finally, allopolyploids often display a greater degree of heterozygosity (Ranney, 2006), low fertility, and low embryonic viability (Soltis and Soltis, 1995; Comai et al., 2000; Schranz and Osborn, 2000). This, however, is not the case in *svt2*. The fact that *svt2* is fertile and that *svt2* offspring with enlarged morphology are being produced suggests that *svt2* is neither a somatic nor a gamete polyploid. Thus, it is therefore unlikely that polyploidy in *svt2* contributes to genome instability. This is supported by a study by Ruffio-Chable and co-workers who reported between 5% and 21% of aberrant plants in F_1 hybrids of *Brassica oleracea*, which was not correlated with abnormal ploidy levels (Ruffio-Chable et al., 2000).

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 breaks (Filkowski et al., 2004). 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* (Filkowski et al., 2004). 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 (Roth et al., 1989; Chen et al., 2009). In the process of soybean cell culture, massive specific changes of genomic loci spread throughout the genome were observed (Roth et al., 1989). It was suggested that this genetic variation is a consequence of specific recombinational events. Similarly, a single-copy 5.7 kilobase DNA fragment that was not present in the parent line, appeared in genotrophs in flax in response to particular growth conditions (Chen et al., 2009).

2.4.3 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, which is often associated with chromosome breaks and causes unstable mutations that 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 (Lisch, 2009). 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 present in its entirety somewhere else in the genome. Transposon termini represent inverted repeats. This, however, is not the case in *svt2* (Fig. 2.6). 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, arguing against the activity of transposons, thereby causing the insertion or loss of novel sequences in *svt2* (Fig. 2.6).

Random mutations caused by EMS mutagenesis may have activated an unknown mechanism in *vtc1-1* seeds, giving rise to the phenome 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 L*er*-0 wild types (Fig. 2.6). The disappearance of the *vtc1-1* mutation in *svt2* (Figs. 2.3d, 2.6) may also be explained by the introduction of a random mutation. Exposure of *vtc1-1* seeds to EMS could have reversed the original *vtc1-1* mutation, which was initially generated by EMS (Conklin et al., 1999), to the wild-type sequence. Interestingly, Conklin and co-workers (1999) previously isolated two *vtc1* alleles, *vtc1-1* and *vtc1-2*, containing the exact same single cytosine to thymine point mutation at 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 (Conklin et al., 1999). 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 (Lukowitz et al., 2001). To date, only the *vtc1-1* (Conklin et al., 1999) and *hsn1* mutations (Qin et al., 2008) have been isolated and reportedly do not cause embryo lethality. This suggests some form of allelic constraint that has been reported in *Arabidopsis* previously (Kreps et al., 1996; Li and Last, 1996). 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, was reported to induce high frequency phenotypic instability in the *Arabidopsis* disease resistance genes, *CPR1* and *BAL*, mapping to the RPP5 locus (Yi and Richards, 2008). 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_2 generation. Phenotypic instability was also observed in *bal* x *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 (Yi and Richards, 2009).

Finally, a combination of unequal crossing over, gene conversion, double-strand breaks, DNA recombination, in conjunction of an RNA cache template may explain the loss and reappearance of DNA sequences in *svt2*. Genome-wide non-Medelian inheritance of extra-genomic information in *Arabidopsis* was reported in the *hothead* (*hth*) *Arabidopsis* mutant (Lolle et al., 2005). 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 (Xu et al., 2007). Lolle et al. postulated that these genetic restoration events are the result of a template-directed process that utilizes an ancestral RNA-sequence cache (Lolle et al., 2005). This hypothesis is supported by observations reported by Xu and co-workers (Xu et al., 2007). 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 underway to provide experimental support for this hypothesis.

2.4.4 Concluding remarks

We have isolated a novel *Arabidopsis* mutant that is capable of restoring genetic information that was not present in the chromosomal genome of the parents. However, we suggest that this ancestral information is present in some cryptic form, allowing plants to access this information under stress conditions. How this genome restoration process is triggered and

accomplished mechanistically remains to be determined. Double strand breaks, DNA recombination, and the activity of an RNA cache may be contributing factors. It is clear that genome restoration could be advantages to plants to survive environmental changes for which the ancestral genes were better adapted. This could also lead to the identification and utilization of new traits in crops. Understanding *svt2* could also provide new knowledge on how *Arabidopsis* ecotypes evolved and diversified, and serve as a model to study non-Mendelian inheritance.

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3 CHAPTER 3: Elucidating the role of the autonomous flowering pathway gene *FCA* in the regulation of ascorbic acid biosynthesis in *Arabidopsis thaliana*

3.1 INTRODUCTION

3.1.1 Flowering pathways in *Arabidopsis thaliana*

In higher plants, the timing of the transition from the vegetative to the reproductive phase is essential to ensure reproductive success. Flowering time is controlled by external and internal factors that are integrated in a complex gene regulatory network that ensures the expression of flowering genes, resulting in flower formation (Corbesier and Coupland, 2005; Jack, 2004). Flowering cues include changes in photoperiod, light, temperature, and internal changes in GA (Koornneef et al., 1998). There are four, partially overlapping pathways which lead to the development of floral tissue: the long-day photoperiodic, gibberellic/phytohormone, vernalization, and autonomous pathways (Fig. 3.1; Boss et al. 2004; Jack 2004). Boss et al. divides these pathways into two groups: those that *promote* the transition to the floral meristem and those that *enable* this transition. The photoperiodic and gibberllic/phytohormone pathways act as floral promoters, while the vernalization and autonomous pathways enable floral development. This categorization is helpful in understanding the epistatic relationships between flowering pathway genes. The floral promotion pathways function to increase levels of floral integrators; but as long as levels of floral repressors (*e.g.* FLC) remain elevated, floral promotion stimuli are blocked from triggering floral development (Boss et al., 2004; Hepworth et al., 2002).

These flowering pathways incorporate environmental and internal cues in a combinatorial manner to cause flowering at an appropriate time. In the facultative long day plant, *Arabidopsis*, the photoperiodic pathway is activated when peak expression of the transcription factor, *CO*, in the leaves coincides with exposure to far red and blue light which stabilize and cause CO to promote expression of the floral integrators *FT* and *SOC1* (Blazquez, 2005; Hepworth et al., 2002; Huang et al., 2005; Putterill et al., 1995; Valverde et al., 2004). *FT* protein moves through the phloem to the shoot apex, where it interacts with the transcription factor FD to activate *AP1*, *SOC1*, and other floral identity genes (Fig. 3.1; Abe et al., 2005; Blazquez, 2005; Huang et al., 2005; Wigge et al., 2005). Phytohormones can also affect flowering time, with the giberellins being the most thoroughly characterized (Davis, 2009). Gibberellins promote flowering

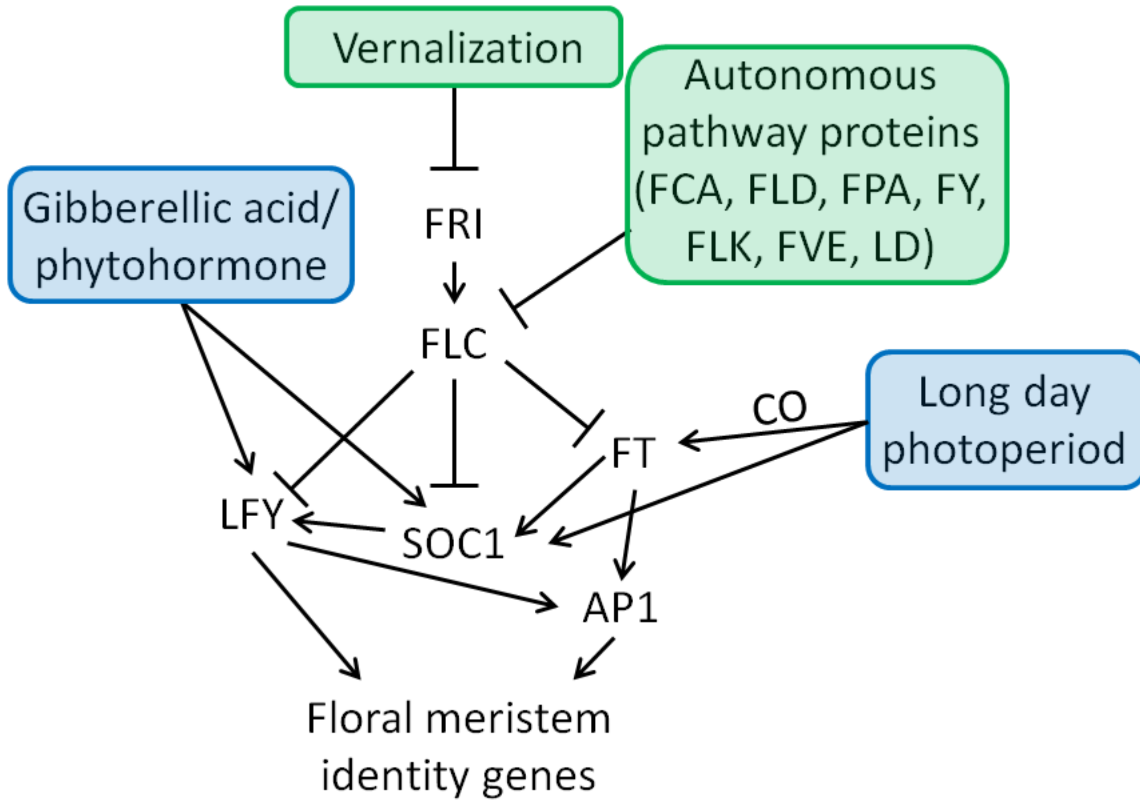


Figure 3.1 Diagram of flowering pathways in Arabidopsis and downstream genes cumulating in floral development.

Pathways promoting floral development are shaded in blue, those that enable floral development are shaded in green. Adapated from Jack (2004) and Blazquez (2005).

through stimulation of the floral meristem identify genes *SOC1*, *LFY*, and *AGL24*; this pathway appears to be important for promotion of flowering during short days, but during long days, its contribution is negligible (Boss et al., 2004; Davis, 2009; Michaels and Amasino, 1999b). Interestingly, it appears that after initial floral promoters are expressed, they then can activate other floral promoters to amplify the transition signal, *e.g.* a positive feedback mechanism to increase expression exists between *SOC1* and *AGL24* and the ability of *LFY* to directly increase *AP1* expression (Fig. 3.1; Jack, 2004; Lee and Lee, 2010; Liu et al., 2008; Michaels et al., 2003). Together, day length and phytohormone production function to upregulate floral meristem identity genes, but these processes rely on the downregulation of floral integrator suppressors *TFL1*, *SVP*, *TOE1/2*, and *FLC* (Boss et al., 2004). In winter annual *Arabidopsis* plants, *FLC* expression is maintained by *FRI* (Michaels and Amasino, 1999a). However, following a vernalization period (i.e. the promotion of flowering by low-temperature treatment), *FLC* expression is suppressed through various chromatin modifications (He et al., 2003; Noh and Noh, 2006; Veley and Michaels, 2008) Signals from these pathways work towards regulating *FLC*, *SOC1*, *FT*, *AP1* and *LFY* (Koorneef et al. 1998; Jack 2004). The floral meristem identity genes direct the conversion from a vegetative to floral meristem and direct formation of lateral floral meristems on the primary inflorescence (Fig. 3.1; Jack, 2004; Kobayashi and Weigel, 2007).

As introduced above, *vtc* mutants exhibit early flowering independent of the photoperiod (Kotchoni et al., 2009). Kotchoni and co-workers undertook a study to identify the flowering pathway that promotes the early flowering phenotype. Through the generation of double mutants with defects in AA biosynthesis and flowering, they identified *vtc1-1 fca-1* that was still delayed in flowering but had substantially recovered AA levels. FCA acts in the autonomous flowering pathway and was therefore hypothesized to possibly contribute to the regulation of AA biosynthesis in Arabidopsis.

3.1.2 The autonomous flowering pathway

The autonomous flowering pathway enables flowering independently of environmental cues (Boss et al., 2004; Quesada et al., 2003). It consists of seven genes which promote floral induction through suppressing *FLC* expression independent of *FRI* and vernalization (Veley and Michaels, 2008). The effect of *FRI* on *FLC* is epistatic to the autonomous pathway genes. Thus, mutations in *FRI* or *FLC* promote flowering, while mutations in the autonomous pathway genes suppress flowering (Veley et al. 2008). It is important to note that while stimuli which promote floral development may be present, the expression of floral repressors act epistatically to control flowering, and it is only when the repressors are downregulated that the meristem is able to switch from vegetative to floral growth (Fig 3.1; Boss et al. 2004).

While the genes of the autonomous pathway have been identified, their mechanism of *FLC* suppression is not fully understood (Veley and Michaels, 2008). The FLD protein of the

autonomous pathway is thought to be a histone demethylase, as it is homologous to the human protein, LSD1, which has been shown to have histone H3 lysine 4 demethylase activity (Shi et al., 2004; Veley and Michaels, 2008). FVE has a human homolog which is suggested to be involved in histone deacetylation (He et al., 2003). Interestingly, *fld* and *fve* mutants have hyperacetylated histone H4 at *FLC* chromatin (although the magnitude of hyperacetylation is not as substantial in *fve* as in *fld* mutants). However, this hyperacetylation is absent in *fca*, *fpa*, or *ld* mutants (He et al., 2003). He et al. (2003) identified a 295-bp region within the first intron of *FLC*, which, when deleted, mimics the hyperacetylated phenotype of *fld* mutants. The LD protein appears to be a nuclear protein and contains glutamine-rich domains characteristic of some transcription factors (Lee et al., 1994). *FCA* encodes a protein with two RNA-recognition motifs (RRMs) and a WW-repeat domain and is alternatively spliced with four splice variants (Fig. 3.2; *FCA*-α, *FCA*-β, *FCA*-γ, and *FCA*-δ) where correct variant processing occurs through interaction with FY using the WW-repeat domain (Macknight, 2002; Macknight et al., 1997; Page et al., 1999; Simpson et al., 2003). *FCA*-γ is the only splice variant which encodes the active FCA protein which associates with *FLC* chromatin (Liu et al., 2007; Macknight et al., 1997). FCA also requires FLD for *FLC* downregulation (Liu et al., 2007) and interacts with the chromatin remodeling factors ATSWI3A and ATSWI3B (Sarnowski et al., 2002 ; Sarnowski et al., 2005). FPA and FCA are both important regulators in RNA-mediated chromatin silencing, both at the *FLC* locus and other loci genome-wide (Baurle et al., 2007). FY is important for correct *FCA* splicing and proper FCA function with null *fy* alleles being lethal (most likely due to FY being a necessary protein in the RNA 3' end processing complex), while hypomorphic alleles are late flowering (Simpson et al., 2003). However, FY has been observed to affect *FLC* expression and polyadenylation in a manner independent of FCA (Feng et al., 2011). In sum, the proteins of the autonomous pathway act through independent and redundant mechanisms to modify the *FLC* chromatin in the promotion of flowering (Veley and Michaels, 2008).

The goal of this study was to identify the mechanism through which the autonomous pathway contributes to the regulation of AA biosynthesis in *vtc* mutants. In order to test this, *vtc fca* double mutants were generated and characterized.

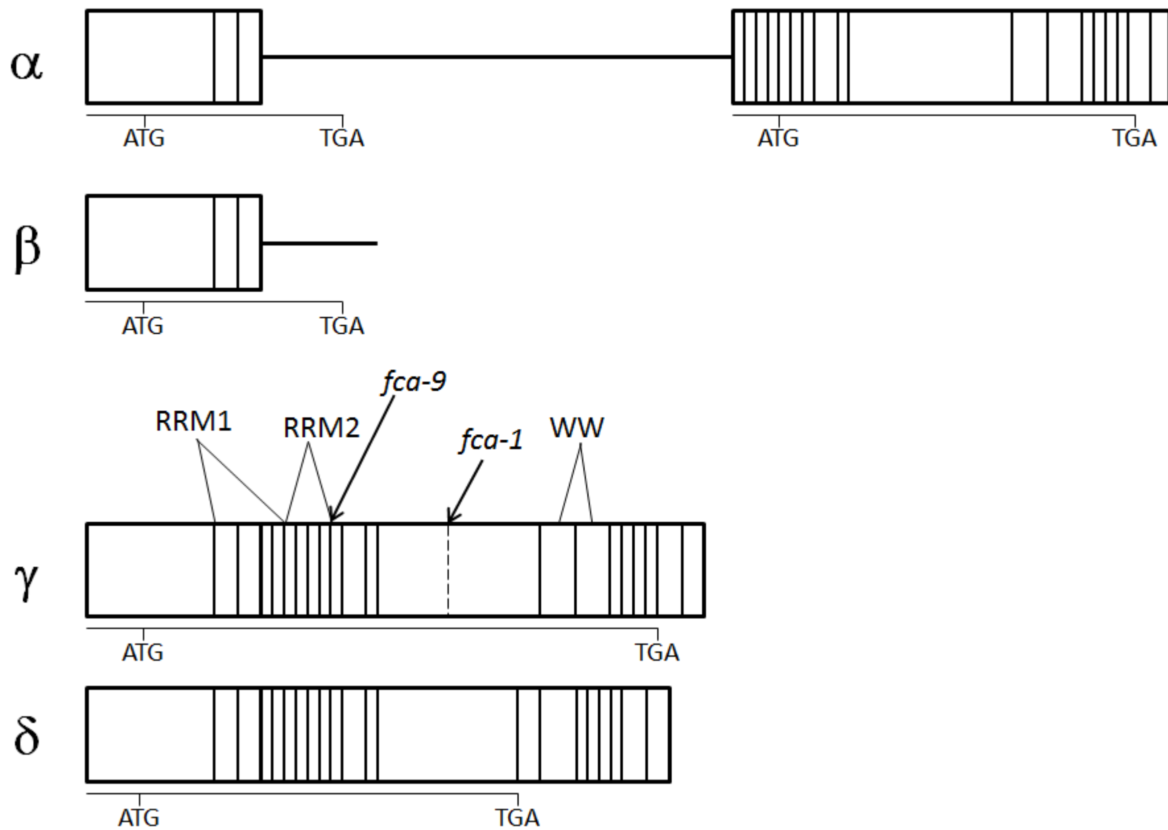


Figure 3.2 Diagram of the four alternative FCA transcripts after Macknight et al. (1997).
Boxes represent exons, the heavy horizontal line represents intron three. Horizontal lines below gene schematics represent possible ORFs within the above transcript. The exon regions encoding the two RRM and WW domains are indicated above the γ transcript (Macknight et al., 1997). The approximate mutation locations for *fca-9* (frame-shift mutation at the end of exon 9 encoding a truncated protein) and *fca-1* (C to T mutation introducing a premature stop codon within exon 13) are shown by arrows on the γ transcript (Feng et al., 2011; Macknight et al., 1997; Page et al., 1999).

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growth conditions

Arabidopsis thaliana L. Heynh wild-type ecotype Columbia-0 (Col) and previously described *Arabidopsis* mutants *vtc1-1*, *vtc2-1*, *vtc3-1*, *vtc4-1* kindly provided by P. Conklin (Conklin, 2001; Conklin et al., 2000; Conklin et al., 1996) were grown in a growth chamber (Percival, Perry, IA). Mutant plants were backcrossed to Col wild type four times (*vtc1-1*), three times (*vtc3-1*) or two times (*vtc2-1*, *vtc4-1*). The flowering time mutants *fca-1* (CS167) and the wild type Landsberg *erecta*-0 (CS20; L*er*) were obtained from the ABRC stock center. The flowering time mutant *fca-9* (Page et al. 1999) was kindly donated by R. Macknight. The *fpa-7* (Michaels and Amasino, 2001), *fld-3* (He et al., 2003), and *fpa-7 fld-3* (Veley and Michaels, 2008) flowering time mutants were kindly provided by S. Michaels. Plants were grown on soil (Metromix 360, BFG Supplies, Burton, OH) in replicate flats, containing 32 inserts with wild-type controls and mutants always present on the same flat. Seeds were suspended in 0.1% phytoblend agar (Caisson Laboratories, Inc.) and stratified at 4°C for 2 to 4 days before planting. Temperature in the chamber was 23°C at day and at night. Plants were grown under long-day (LD, 16 h light/8 h dark, growth chamber lights turned on at 6:00 AM and turned off at 10:00 PM) at a light intensity of 160 µmol photons m^{-2} s^{-1} (fluorescent bulbs). Whole rosettes of three-week-old plants were harvested for RNA extraction, AA and ABA content measurements, shock frozen in liquid nitrogen, and stored at -80°C until further analysis.

3.2.2 Root length measurements

For assessment of root growth, seeds were surface-sterilized and grown on 1x Murashige and Skoog (MS) medium in omni trays as described in (Barth et al., 2010). Trays were sealed with one layer of micropore tape (3M), put in vertical orientation, and placed in the growth chamber using the growth conditions described above. Each plate contained wild type and mutant seed. Root length was measured in seven-day-old seedlings using a ruler.

3.2.3 Generation and identification of *vtc* and flowering time double mutants

The *vtc1-1* mutant (Col background; Conklin et al. 2000) was crossed with *fca-1* (L*er* background; Macknight et al., 1997), and *fca-9* (Col background; Page et al., 1999), respectively. The *vtc4-1* mutant (Col background; Conklin et al., 2000) was crossed to *fca*-9. F_1 progeny of the crosses were allowed to self. F_2 progeny were screened for AA deficiency using the nitroblue tetrazolium assay (Conklin et al., 2000) and DNA was extracted from progeny that scored as AA-deficient. Sequencing was conducted to determine plants homozygous for both mutations using primers listed in Table 3.1. F_3 seeds from homozygous double mutants were used for experiments. The individual Col and L*er* wild types and Col/L*er* crosses were used as controls.

Table 3.1 Sequences of oligonucleotide primers used for qRT-PCR and sequencing.

Primer Name	5` 3`	ATG Number
VTC1-F	TCGCTTGAGACCATTGACT	AT2G39770
VTC1-R	TCGCTAGAGCCAGAGGAC	
VTC4-F	TGTAAGCCGCAACGCCTCAG	AT3G02870
VTC4-R	AGCAGCTCTTTCCCAATCACA	
TUB2-RT-F2	CTCAAGAGGTTCTCAGCAGTA	AT5G62690
TUB2-RT-R2	TCACCTTCTTCATCCGCAGTT	
FCA-9-F1	ATGCGGGATGAATATAGACAGAG	AT4G16280
FCA-9-R1	CCAACAGGAGGTGCCATTTC	
FCA-1-F1	GCCTATGGGGTTTGCCTACGATGA	
FCA-1-R1	CTGACCGGAAAGAGGCTGCTGGAC	

To minimize genetic variability, resulting from crossing two different backgrounds, seeds were pooled from 12 different crossing events of the Col and L*er* wild-type controls (Col/L*er*; (Miller et al., 2007) and at least three independent double mutants were evaluated for flowering time.

3.2.4 Qualitative AA content analysis in double mutants

A total of 183 F_2 plants *vtc1-1 fca-1* from three independent crosses (Kotchoni et al., 2009) were assayed for AA content using the nitroblue tetrazolium assay (Conklin et al., 2000). Plants were scored as positive (+), i.e., wild-type content of AA, negative (-), i.e., AA-deficient, or positive/negative (+/-), i.e., intermediate AA content. Recessive trait heredity was calculated using qualified AA levels in crosses to see if they agreed with Mendelian inheritance laws. Statistical analysis was conducted using a X^2-test (α = 0.05).

3.2.5 RNA isolation, cDNA synthesis, and gene expression analysis

Total RNA was extracted from rosette leaf tissue collected and pooled from seven-day-old seedlings using the Tri-Reagent protocol (Molecular Research Center, Inc., Cincinnati, OH). One microliter of total RNA was treated with 1 µL of DNaseI (Invitrogen, Carlsbad, CA) and subsequently used for reverse transcription using 10 pg of oligo(dT) primers using the first-strand cDNA synthesis kit (Invitrogen). Quantitative RT-PCR reactions were set up using 2.5 pmole of gene specific primers and 1:10 dilutions of cDNA:DNase/RNase free water with the iQ SYBER Real-Time Master Mix (Bio-Rad, Hercules, CA) in a total volume of 10 µL. Negative controls contained water instead of reverse transcriptase. Reactions were run in a Bio-Rad iCycler for 40 cycles. The threshold cycles (C_T) were calculated using iQ software (Bio-Rad) and relative transcripts (RT) were calculated using the formula: RT = $1/2^{CT}$. The RT values of the genes assessed were normalized to *TUBULIN2* and mean values of biological replicates were calculated. Experiments were performed at least three times. PCR fragments were separated on a 1% agarose gel stained with ethidium bromide to check for correct fragment amplification. Gene-specific primer sequences are listed in Table 3.1.

3.2.6 Ascorbic acid quantification

Leaf AA content was determined in leaves from three-week-old rosettes using the iron reduction assay (Dowdle et al., 2007; Mukherjee et al., 2010).

3.2.7 Abscisic acid quantification

For abscisic acid (ABA) content measurements, tissue (0.1-0.15 g) was crushed in liquid nitrogen, extracted in 1 ml 80% (v/v) acetone, and incubated overnight in the dark at 4ºC. Samples were centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was diluted 1:10 with Tris-buffered saline (pH 7.5). Abscisic acid content was determined in diluted samples using the Phytodetek ABA kit (Agdia, Elkhart, IN) following the manufacturer's protocol.

3.2.8 Statistical analysis

Data presented are mean values $\pm$ standard errors (SE). Experiments were repeated at least three times. *P* values were determined by Student's *t*-test or X^2-test analyses, as indicated.

3.3 RESULTS

3.3.1 AA levels are partially recovered in *vtc fca* double mutants along with some but not all *vtc* pleiotropic phenotypes

The *vtc1-1 fca-1* double mutant, which is in the Col/L*er* background, has a partially recovered AA content (Fig. 3.2). The total AA content increased more than twice in the double mutant compared to the *vtc1-1* single mutant and is approximately 70% of the Col/L*er* wild-type control and the *fca-1* single mutant. To test whether a similar recovery of AA content occured in the other *vtc* mutants and whether this effect is *fca* allele-specific, we crossed *vtc1-1* and *vtc4-1* mutants with the *fca-9* mutant. This also had the advantage that the double mutants were then entirely in the Col background. We then determined the AA content of the homozygous double mutants. The AA level of the *vtc1-1 fca-9* and *vtc4-1 fca-9* mutants is partially, but to a much lower extent, recovered compared to their respective AA deficient single mutants (Fig. 3.3).

Furthermore, we evaluated whether the high level of ABA detected in *vtc* mutants would decrease to wild-type ABA levels in the *vtc fca* double mutants. As shown in Fig. 3.4, ABA content decreased dramatically in the *vtc1-1 fca-1* double mutant, although it was still slightly higher in the Col/L*er* wild-type control.

Compared to the respective wild-type controls, root development is partially recovered in the *vtc1-1 fca-1* double mutant, but this is not the case in the *vtc1-1 fca-9* double mutant when grown in the presence of ammonium (Fig. 3.5). These data suggest that *fca* does neither act in an allele- nor in a gene-specific manner. It is possible that combining two different backgrounds (*vtc1-1* in Col-0, *fca-1* in L*er*-0 background) may be the reason for the elevated AA content in the *vtc1-1 fca-1* double mutant and other restored phenotypes. However, strong effects in the Col/L*er* wild-type control are not observed. Therefore, it is not clear what is causing the recovered phenotype in *vtc1-1* in the presence of the *fca-1* allele.

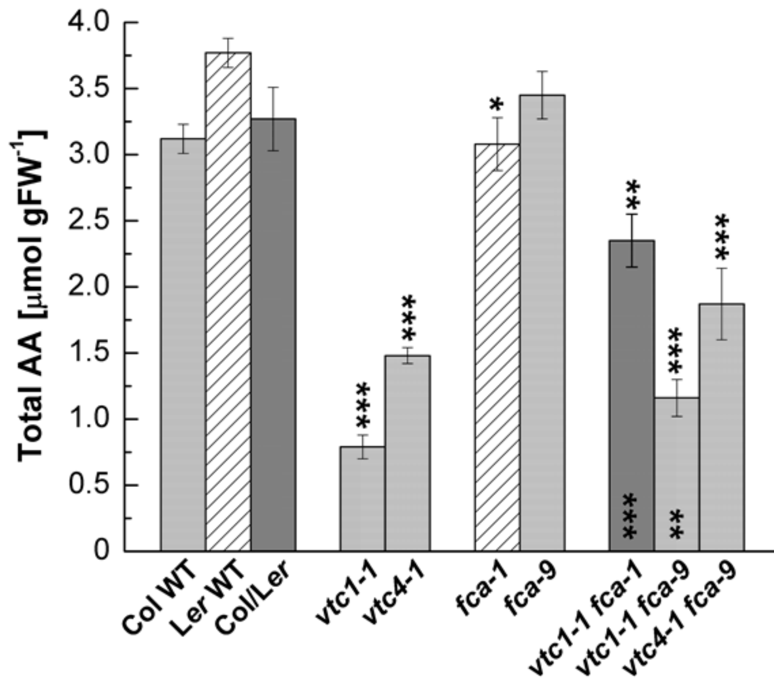


Figure 3.3 Mean ascorbic acid (AA) content in three-week-old rosette leaves of *vtc* and *fca* single and double mutants compared to Col, L*er* and Col/L*er* wild-type controls.

Bars represent means ± SE. Asterisks indicate * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test. Statistical differences above bars represent difference from the respective WT, while statistical differences directly above the x-axis represent differences from the respective *vtc* single mutant. Bar shading indicates the lines of the same genetic background.

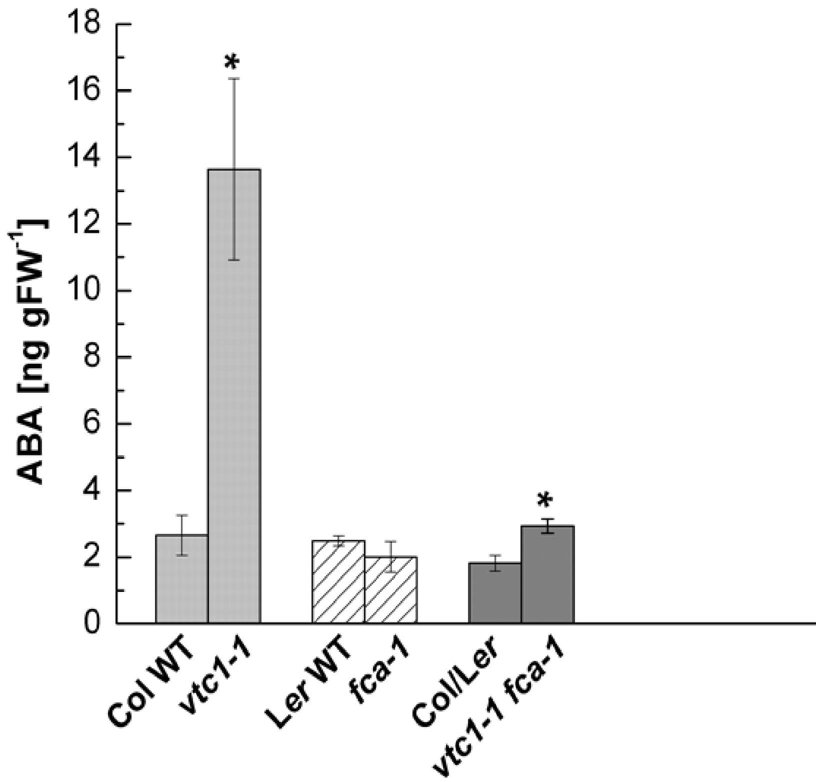


Figure 3.4 Total abscisic acid (ABA) content in Col, L*er* and Col/L*er* wild-type controls compared to *vtc1-1* and *fca-1* single mutants and the *vtc1-1 fca-1* double mutant.

Bars represent means ± SE. Asterisks indicate significant differences compared to the respective wild-type controls. * *P*-value < 0.05, Student's *t*-test. Bar shading represents lines of the same ecotype.

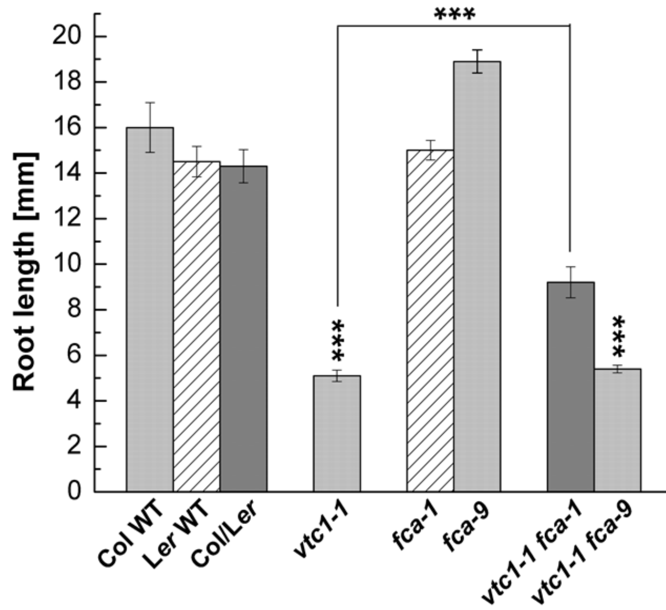


Figure 3.5 Primary root length in seven-day-old Col, L*er*, Col/L*er* wild-type controls, *vtc1-1*, *fca-1*, *fca-9* single and *vtc1-1 fca-1* and *vtc1-1 fca-9* double mutants grown on 1x MS medium.

Bars represent means ± SE. Asterisks designate significant differences between mutants and respective wild-type controls, unless otherwise indicated. * P-value < 0.05, *** P-value < 0.005 Student's t-test. Bar shading indicates lines of the same genotype.

3.3.2 *VTC* transcripts are not altered in *vtc fca* double mutants

We hypothesized that suppression of AA deficiency occurs through increased transcription of the *VTC* genes in *vtc fca* double mutants (Fig. 3.8). Using quantitative real-time PCR (qRT-PCR) we analyzed *VTC1* and *VTC4* mRNA in *vtc1-1 fca-1*, *vtc1-1 fca-9* and *vtc4-1 fca-9* double mutants, respective single mutants, and wild-type controls. In *vtc1-1*, transcripts of *VTC1* were expected to be approximately at the wild–type level since the mutation affects enzyme activity and not transcription (Conklin et al., 1999). This is indeed the case (Fig. 3.6a). Furthermore, *VTC1* mRNA levels are similar in the double mutants and Col wild type. Interestingly though, *VTC1* transcript levels are substantially lower in the L*er* and Col/L*er* wild types and the *fca-1* single mutant (Fig. 3.6a). Transcript levels of *VTC4* were similar in all genotypes, except in the L*er* wild type, which contained somewhat higher *VTC4* mRNA levels (Fig. 3.6b). This data suggests that in the presence of *fca* mutations, transcript levels of *VTC1* or *VTC4* are not affected.

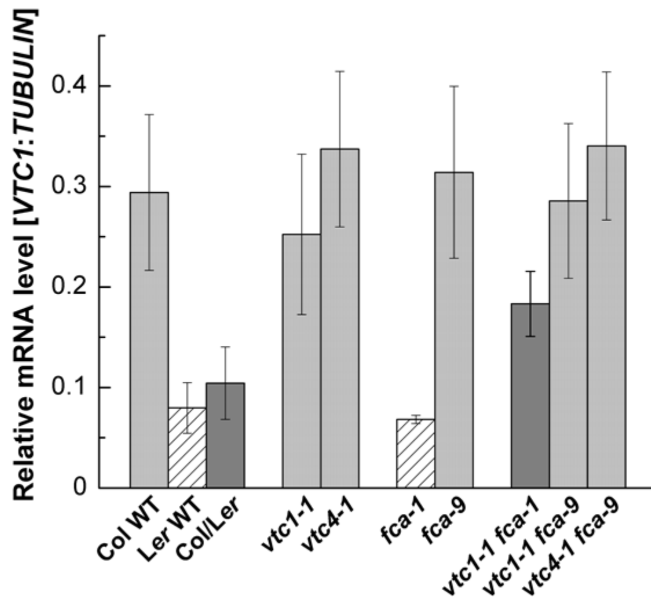
3.3.3 Ascorbic acid levels in autonomous pathway flowering time mutants *fca-1*, *fca-9*, *fld-3*, *fpa-7*, and *fpa-7 fld-3* are unchanged

Because FCA interacts with other proteins in the autonomous flowering pathway, we hypothesized that one or more of these interacting proteins may act at the *VTC1* locus or other loci encoding enzymes in the D-mannose/L-galactose pathway to suppress AA biosynthesis. If this were the case, we would expect to see elevated AA amounts in mutants that are affected in the autonomous flowering pathway. As is illustrated in Fig. 3.7, the AA content in *fca-1*, *fca-9*, *fld-3*, *fpa-7*, and *fpa-7 fld-3* is comparable to the wild type. This data suggest that mutations in these autonomous flowering pathway genes do not alter the AA content.

3.4 DISCUSSION

3.4.1 The autonomous flowering pathway protein, FCA, does not appear to be involved in transcriptional regulation of the *VTC1* and *VTC4* genes

Kotchoni et al. (2009) demonstrated that the *vtc* mutants have significantly reduced levels of *FLC* mRNA during long days, but this was not directly attributable to the concentration of AA, as *vtc1-1 fca-1* mutants displayed a delayed flowering phenotype despite having approximately 70% of wild-type AA. They also observed that double mutants between *vtc1-1* and circadian clock and photoperiodic pathway genes exhibited delayed flowering and reduced AA content, and *vtc* mutants did not have increased mRNAs of gibberellin biosynthesis genes. These data indicate that AA does not directly appear to influence flowering directly through the defined flowering pathways. The data presented here do not suggest that the early flowering phenotype in the *vtc* mutants is caused by interaction of the examined autonomous



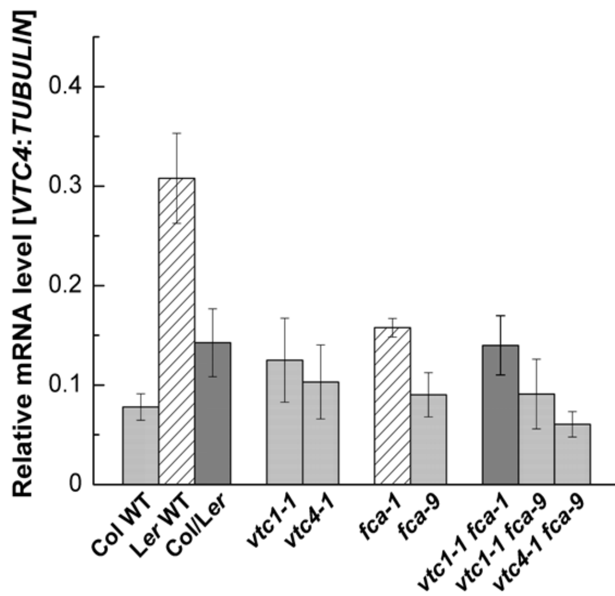


Figure 3.6 Relative transcript levels of *VTC1* (a) and *VTC4* (b) in mutants and respective controls normalized to *TUBULIN* mRNA.

Bars represent means ± SE. No statistical significance was observed, $n = 3$ Student's t-test, $P < 0.05$. Bars of the same shading represent lines of the same genotype.

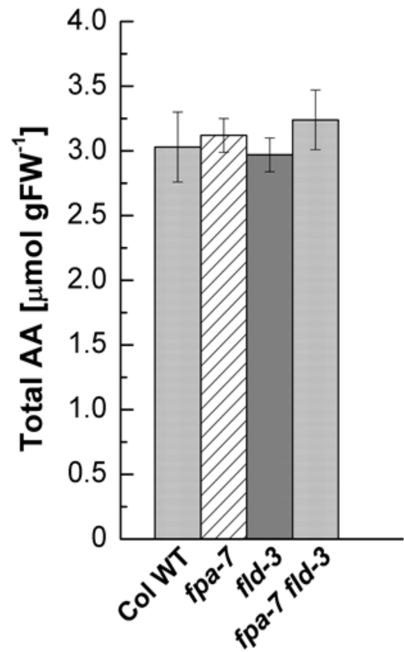


Figure 3.7 Total ascorbic acid (AA) content in three-week-old rosette leaves of autonomous flowering pathway mutants compared to Col wild type (Col WT).

Bars represent means ± SE. No statistical significance was observed, Student's *t*-test, $P < 0.05$.

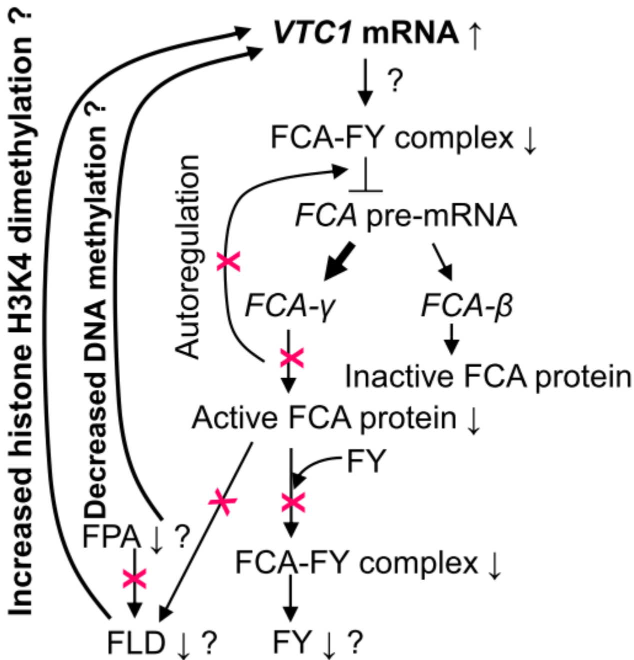


Figure 3.8A hypothetical model of the interaction between VTC1 and FCA in the *vtc1-1 fca-1* double mutant.

Arrows pointing up or down illustrate an increase or decrease, respectively. Red crosses indicate dysfunction. Since FCA requires both FY and the demethylase FLD, which requires FPA, we propose that the defect in FCA results in decreased FLD and FPA activities. FLD is required for histone H3K4 demethylation. Thus, H3K4 dimethylation is increased in *fca* mutants, which is associated with overall increased transcriptional activity. In contrast, DNA methylation is compromised in *fca* and *fpa* mutants. Hence, we hypothesize that *VTC1* transcription is enhanced in the double mutant through increased H3K4 dimethylation and/or decreased DNA methylation. It is not clear why *fca-1* single mutants do not have higher than wild-type levels of AA. We suggest that there are additional factors acting in this regulation and that negative feedback control by AA itself (not indicated) is contributing to maintaining AA homeostasis.

flowering pathway proteins at transcriptional regulation of the *VTC1* or *VTC4* loci. We do provide evidence that mutations in the *FCA* gene suppress AA deficiency in the *vtc1-1* and the *vtc4-1* mutants in a recessive manner (Fig. 3.3). This suggests that in the wild type, FCA functions to limit AA accumulation, presumably in order to maintain AA homeostasis. This is most likely does not occur directly through FCA, but may be through one of its interacting partners by an unknown mechanism. Our initial hypothesis proposed that FCA and/or one of its interacting partners may affect chromatin modifications at the *VTC* loci as mutations in *FCA* and *FPA* suppress RNA-mediated chromatin silencing (Baurle et al., 2007). However, our qRT-PCR data do not support a role of FCA in the transcriptional regulation of *VTC* genes (Fig. 3.6). Since the *FCA* pre-mRNA is a target for alternative splicing (Macknight et al., 1997; Page et al., 1999; Simpson et al., 2004), it is possible that the *VTC* genes (or genes within other AA biosynthetic pathways) are also targets of the *FCA*-splicing complex (Fig. 3.8). Currently listed on The Arabidopsis Resource (http://arabidopsis.org) are two known *VTC1* transcripts which have an alternatively spliced 3’ UTR region and three possible alternative transcripts for *VTC4*. Interestingly, using the Arabidopsis Small RNA Project database (http://asrp.cgrb.oregonstate.edu/db/; Backman et al., 2008; Gustafson et al., 2005), we identified two small RNAs which match the *VTC1* gene sequence. One of these matches a 20 base-pair region in the fourth exon and the other matches a 22 base-pair sequence near the 5’ region of the 3’ UTR. Considering the RNA-binding properties of FCA and the importance of FY in RNA processing—null *fy* mutant alleles are embryo lethal (Henderson et al., 2005)—it seems plausible that the *VTC* genes may be regulated at the post-transcriptional level, but this needs further experimental evidence.

The two *fca* mutant alleles, *fca-1* (L*er*-0 background) and *fca-9* (Col-0 background), have varying effects on the suppression of *vtc* mutations. Mixing the Col and L*er* backgrounds in the *vtc1-1 fca-1* double mutant may complicate interpretations due to the presence of polymorphisms and modifier genes that may affect the AA phenotype (Koornneef et al., 2004). Supporting this idea are the results from a microarray experiment conducted by Marquardt et al. (2006) that aimed to find additional targets of *FCA* by comparing expression changes in Col-0, *fca-9*, L*er*-0, and *fca-1*. The group observed many expression changes in an unexpected accession-specific manner. However, the authors speculated that the observed differences could be due to inter-experimental variation that could not be overcome through their analyses or that genome-sequence differences interfered with probe hybridization (Marquardt et al., 2006). The *VTC1* gene does have polymorphisms between Col-0 and L*er*-0, although they appear to neither affect *VTC1* transcript accumulation (Fig. 3.6) nor AA biosynthesis (Fig. 3.3). Therefore, the question arises whether *fca-1* and *fca-9* mutant alleles differ in their strength. The *fca-1* mutant contains a premature stop codon within exon 13, which leads to the loss of the WW domain (Fig. 3.2; Macknight et al., 1997). The *fca-9* mutant is a splice mutant that

produces a truncated protein even shorter than that in *fca-1* (Fig. 3.2; Page et al. 1999). In respect to flowering, the relative strength of the *fca-1* and *fca-9* mutations cannot be compared, because the Col-0 ecotype contains dominant alleles at *FLC*, enhancing the late flowering phenotype of *fca* mutations (Sanda, 1996). Although, in support of accession-specific transcriptome differences, *fca-9* and *vtc1-1 fca-9* double mutants flower much later than *fca-1* and *vtc1-1 fca-1* double mutants (Kempinski and Barth, unpublished results).

3.4.2 A wide variety of control mechanisms affect the autonomous and other flowering pathways in a combinatorial manner, making it difficult to elucidate the manner in which ascorbic acid biosynthesis is affected by flowering time

Plants must incorporate both environmental and internal cues to switch from vegetative to floral growth. Multiple pathways (Fig. 3.1), which to some extent are reglated by epigenetic mechanisms, mediate the transition from the vegetative to the reproductive phase (Jack, 2004), requiring regulatory proteins. Proteins involved in the autonomous pathway can be divided into two broad groups: RNA-binding proteins (including FPA and FCA; Macknight et al., 1997; Schomburg et al., 2001) and chromatin-modifying proteins (Noh and Noh, 2006). In conjunction with regulating flowering through the autonomous pathway, several of these proteins also act redundantly to alter gene expression of non-flowering associated genes (Veley et al. 2008). Baurle et al. (2007) found that FCA and FPA act redundantly with each other, and in parallel with siRNA silencing machinery, to suppress many loci across the genome whose silencing is dependent on siRNAs and are not directly linked to floral induction. As stated above, it could be possible that the *VTC* genes are post-transcriptionally regulated or that flux through another AA biosynthetic pathway could be affected (at least, partially) by FCA and its partners. The study by Kotchoni et al. (2009) showed that AA does not appear to directly affect any specific flowering pathway and it seems likely that the pleiotropic phenotypes (Table 1.1) of the *vtc* mutants contribute in a complex manner to the early flowering phenotype. For example: elevated ABA is known to delay flowering, while elevated SA can suppress *FLC* under short days, but independently of *FCA* (Davis, 2009).

3.4.3 Concluding remarks

Taken together, it has been demonstrated that FCA has other targets beyond floral induction (Marquardt et al. 2006). We have found that FCA influences the accumulation of AA, a process that does not involve transcriptional regulation of *VTC* genes. It is possible that FCA and its partners act at one or more of the other AA biosynthetic pathways (Hancock and Viola, 2005a; Hancock and Viola, 2005b; Lorence et al., 2004). We speculate that FCA and its interacting partners are important for maintaining AA homeostasis. Given the current literature, this may occur through multiple partners regulating post-transcriptional processes.

This might also explain why it is difficult to elevate AA content in plants substantially through overexpression of AA biosynthetic genes (Ishikawa et al., 2006). It is clear that there is link between AA accumulation and flowering time and this work provides additional groundwork for elucidating the role of AA in flowering time.

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