Identification of Arabidopsis VTC3 as a putative and unique dual function protein kinase::protein phosphatase involved in the regulation of the ascorbic acid pool in plants

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Abstract
Ascorbic acid (AsA) is present at high levels in plants and is a potent antioxidant and cellular reductant. The major plant AsA biosynthetic pathway is through the intermediates d-mannose and l-galactose. Although there is ample evidence that plants respond to fluctuating environmental conditions with changes in the pool size of AsA, it is unclear how this regulation occurs. The AsA-deficient Arabidopsis thaliana mutants vtc3-1 and vtc3-2 define a locus that has been identified by positional cloning as At2g40860. Confirmation of this identification was through the study of AsA-deficient At2g40860 insertion mutants and by transgenic complementation of the AsA deficiency in vtc3-1 and vtc3-2 with wild-type At2g40860 cDNA. The very unusual VTC3 gene is predicted to encode a novel polypeptide with an N-terminal protein kinase domain tethered covalently to a C-terminal protein phosphatase type 2C domain. Homologues of this gene exist only within the Viridiplantae/Chloroplastida and the gene may therefore have arisen along with the d-mannose/l-galactose AsA biosynthetic pathway. The vtc3 mutant plants are defective in the ability to elevate the AsA pool in response to light and heat, suggestive of an important role for VTC3 in the regulation of the AsA pool size.

Key words: Antioxidant, Arabidopsis, ascorbic acid, biosynthesis, kinase, phosphatase, vtc3, Smirnoff–Wheeler.

Introduction
Ascorbic acid (AsA) is of vital importance to the health of both animals and plants. Historically and to a large part today, plants provide this essential vitamin (vitamin C) in the human diet. Plants accumulate AsA to very high (mM) concentrations. Acting as an antioxidant, it provides plants with protection against a wide variety of biotic and abiotic environmental stresses (Gallie, 2013). In addition, plants require AsA as a cofactor for a number of mono- and dioxygenases for cell-wall biosynthesis (de Pinto and De Gara, 2004) and as a redox status indicator involved in the activation of processes such as pathogen defence (Pavet et al., 2005), flowering time (Barth et al., 2006; Kotchoni et al., 2009), and stomatal closure (Chen and Gallie, 2004).

Substantial progress has been made in understanding AsA biosynthesis in plants. In 1998, Wheeler et al. (1998) proposed a pathway in which AsA is synthesized via d-mannose and l-galactose: D-glucose → d-glucose-6-P → d-fructose-6-P → d-mannose-6-P → d-mannose-1-P → GDP-d-mannose → GDP-l-galactose → l-galactose-1-P → l-galactose → l-galactono-1,4-lactone → l-ascorbate.

Much additional biochemical and molecular genetic evidence now exists in support of the Smirnoff–Wheeler (SW) pathway, and genes encoding all of the proposed biosynthetic enzymes have been identified in higher plants (Østergaard et al., 1997; Imai et al., 1998; Conklin et al., 1999, 2006; Wolucka et al., 2001; Gatzek et al., 2002; Dowdle et al., 2007; Laing et al., 2007; Linster et al., 2007; Maruta et al., 2008).
Synthesis of AsA via alternative pathways such as l-gulose (Wolucka and Van Montagu, 2003) or D-galacturionate (Agius et al., 2003) has also been suggested. Although ascorbate pool size varies with environmental conditions (Panchuk et al., 2002; Dowdle et al., 2007; Hoebberchts et al., 2008) and developmental stage (Conklin et al., 2000; Ioannidi et al., 2009; Zhang et al., 2009; Badejo et al., 2012), there have been no systematic studies on the control of AsA biosynthesis.

It is well documented that the level of AsA is light dependent (Hirai et al., 1995; Gatzek et al., 2002; Chen and Gallie, 2004; Dowdle et al., 2007). Indeed, a light-responsive cis element has recently been identified in the promoter of the VTC2 gene (encoding GDP-l-galactose phosphorylase; Gao et al., 2011). Both the quality and intensity of light appear to be important in this light regulation. In bean leaves, a high red:far red light regime correlated with an elevation of AsA (Bartoli et al., 2009). Inhibition of photosystem II dramatically reduced the magnitude of the total AsA increase, suggesting that photosynthesis is necessary to achieve high levels of total AsA (Yabuta et al., 2008). The steady state level of transcripts encoding several of the AsA SW biosynthetic enzymes have been shown to correlate with exposure to light and correlate with AsA content (Dowdle et al., 2007; Maruta et al., 2008; Yabuta et al., 2008). However, in a systematic experiment that measured the activities of all the AsA biosynthetic enzymes, only the activity of GDP-l-galactose phosphorylase was found to be responsive to light (Dowdle et al., 2007). l-Galactose dehydrogenase activity may also be light stimulated (Gatzek et al., 2002).

It is becoming increasingly clear that plant AsA biosynthesis regulation is multifaceted, occurring at many levels and in response to several stimuli. Only one gene has been described to-date that appears to play a role, apparently via negative regulation. The Arabidopsis AMR1 (At1g65770) gene product contains a predicted F-box domain, so AMR1 is likely to function via the ubiquitin-proteosome pathway. Expression of this gene is negatively correlated with the transcript abundance of six of the SW pathway genes (Zhang et al., 2009). AMR1 expression is also lower at higher light intensities and in younger plants, both conditions in which AsA levels are elevated (Zhang et al., 2009). The mechanism of AMR1 regulation has not yet been elucidated.

The AsA-deficient Arabidopsis vitamin c (vtc) mutants define four loci (VTC1, VTC2, VTC3, VTC4 (Conklin et al., 1996, 2000) involved in the synthesis of AsA. VTC1, VTC2, and VTC4 encode the aforementioned AsA biosynthetic enzymes GDP-β-mannose pyrophosphorylase (Conklin et al., 1999), GDP-l-galactose phosphorylase (Dowdle et al., 2007; Linster et al., 2007), and l-galactose-1-P-phosphatase (Conklin et al., 2006; Laing et al., 2007). These vtc mutants have been quite useful in the validation of the SW AsA biosynthetic pathway and the study of its regulation and the effect of AsA deficiency on plant physiology. The only VTC gene of unknown function is VTC3. Similar to mutations in the other VTC genes, VTC3 mutations are pleiotropic, rendering the mutant plant less able to synthesize anthocyanin (Page et al., 2012), enhancing the growth defect of an autoimmune response mutant (smel; Zhu et al., 2013), and causing elevated sensitivity to salt-induced photooxidation (Smirnoff, 2000).

Here is described the identification of the VTC3 gene product as a novel dual-function polypeptide predicted to harbour an N-terminal protein kinase domain and a C-terminal type 2C protein phosphatase (PP2C) domain. This gene is highly conserved across the Viridiplantae and is likely involved in the regulation of AsA biosynthesis.

**Materials and methods**

**Genetic mapping of the VTC3 locus**

The VTC3 locus was found to be located ~2 cM centromere distal from microsatellite marker nga168 as previously described (Conklin et al., 2000). A population of 2900 F2; individuals derived from a cross between VTC3/VT3 (Landsberg erecta ecotype) and vtc3-1/vtc3-1 (Columbia-0 ecotype; Col-0) were utilized in the fine-scale genetic mapping. The VTC3-specific genotype of each recombinant was verified in the F3 generation. The InDel (8/8; CER459190; Coreen Arabidopsis Polymorphism Collection, Jander et al., 2002) that defined the closest centromere distal breakpoint was amplified using the primers whose sequences are reported in Supplementary Table S1 (available at JXB online). This marker is located at ~70 kb on BAC F13E7. The microsatellite on BAC T7D17 (chromosome 2, from 16986782 to 16987027) that defined the closest centromere proximal recombination breakpoint was amplified using primers reported in Supplementary Table S2. All amplifications in this study were performed using a PCR Express thermocycler (ThermoElectron, Waltham, MA, USA) unless stated otherwise.

**Sequencing of the VTC3 locus**

Twenty candidate genes were located within the recombination breakpoints that define the VTC3 locus. Candidate genes were prioritized for sequence analysis based on their annotation. For example, genes expressed primarily in the roots and not in the aerial tissues were not immediately considered. Genes annotated as being involved in signal transduction were given first priority for sequencing. Total DNA was extracted from wild-type (Col-0), vtc3-1, and vtc3-2 genotypes using a cetyltrimethylammonium bromide method as previously described (Lukowitz et al., 2001). The At2g40860 locus was amplified and then sequenced from these DNAs using a set of PCR primer pairs that amplified overlapping sequences (Supplementary Table S2). Sequencing was carried out by the Cornell Life Sciences Core Laboratories Center (Ithaca, NY, USA).

**Transgenic complementation analysis**

A full-length pUNI-based At2g40860 cDNA (G11316) from the SSP Consortium (Yamada et al., 2003) was obtained from the Arabidopsis Biological Resource Center. The open reading frame was moved from the pENTR223-1-fsi plasmid to the pMCD2 plant transformation vector (Curtis and Grossniklaus, 2003) with LR cloning II (Invitrogen, Life Technologies). The resultant pEx-VTC3 construct was then used to transform Agrobacterium tumefaciens GV3101 and was introduced into Col-0 wild type, vtc3-1, and vtc3-2 by the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were selected by hygromycin (25 μg ml⁻¹) on 0.5 × Murashige and Skoog medium/1% phytagar. After 2 weeks, hygromycin-resistant seedlings were transplanted to soil. Two-week-old hygromycin-resistant T2 plants from selfed individual T1 lines were pooled (three or four individuals per sample) and assayed for total AsA as described below. Hygromycin-resistant siblings of these T2 individuals were confirmed as transgenic by PCR using primers specific to the 35S promoter of the transgene (Supplementary Table S3).
Subcellular localization

Using Gateway cloning, the above-mentioned At2g40860 cDNA was cloned into the pEarleygate 104 vector (Earley et al., 2006) to create a construct encoding an N-terminal YFP::VTC3 fusion whose expression is driven by the 35S CaMV promoter. This pEG104-cVTC3 plasmid was then transformed into Agrobacterium tumefaciens GV3101 and introduced into Col-0 wild-type plants by the floral dip method. Glufosinate-resistant T1 transformants were selected on soil by spraying with 300 μM glufosinate ammonium. Three-week-old glufosinate-resistant individuals were imaged using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems). Images were obtained sequentially and then superimposed. Chlorophyll autofluorescence was detected with excitation at 488 nm with emission at 672–690 nm while YFP was excited at 488 nm with emission at 526–609 nm.

Identification/verification of At2g40860 insertion mutant lines

Segregating T4 generation seed for the Salk 095743 insertion line (Sessions et al., 2002) was obtained from the Arabidopsis Biological Resource Center. Total DNA was isolated from 22 individuals using the above-described CTAB method. At2g40860 was amplified from these DNAs using gene-specific primers designed to flank the insertion site (Supplementary Table S3). The lines that did not amplify with these wild-type allele-specific primers were then amplified using the insertion allele primer Lbb1.3 (http://signal.salk.edu/tdnaprimers2.html) and the above R flanking primer. Four homozygous insertion individuals were identified. To confirm the site of the insertion, the PCR product obtained from the amplification of one of these lines with Lbb1.3 and the R flanking primer was purified and sequenced using primer Lbb1.3 as the sequencing primer.

T5 generation homozygous mutant seed of the Salk 006988C line from the Salk Homozygous T-DNA Collection was obtained from the Arabidopsis Biological Resource Center. DNA from three individuals was isolated. The line was verified as being homozygous mutant by PCR and sequencing, using the methods described immediately above but using gene-specific primers instead (Supplementary Table S3).

Measurement of AsA content

Acidic extracts were prepared from either whole rosettes or fully expanded leaves for the assay of total and reduced ascorbate using an ascorbate oxidase-based spectrophotometric assay as previously described (Conklin et al., 1996, 1997).

Plant growth conditions

Plants utilized for the measurement of total AsA were grown from seed in Cornell mix (Landry et al., 1995) plus Osmocote (Scotts) in a Conviron growth chamber (23 °C, relative humidity 50%) with a 16/8 light/dark cycle at a photosynthetic photon flux density (PPFD) of 200 μmol m⁻² s⁻¹. Transgenic lines first selected on plates were transplanted into Cornell mix and grown under the same conditions. For measurement of redox status, GalLDH activity and the effect of light on total AsA, plants were grown with a 16/8 light/dark cycle under metal halide bulbs (150 μmol m⁻² s⁻¹) in a commercial soilless mix (Promix BX, Premier Horticuture, Quakertown, PA, USA). Plants subjected to a dark incubation followed by continuous light were subsequently moved into an environmental growth chamber (Percival AR36L3) for 24 h in the dark (21 °C, 70% relative humidity) prior to commencement of continuous light exposure. Control plants remained in the same chamber, but were covered with light-tight cardboard.

The plants subjected to elevated temperature were first grown in the environmental growth chamber for 2 weeks under a 12/12 light dark cycle at 16 °C with 70% relative humidity under fluorescent bulbs at 150 μmol s⁻¹ m⁻² PPFD. For the following 4 days, the plants were grown at 30 °C in continuous light but otherwise under the same conditions.

Results

Identification of VTC3 as At2g40860

The allelic mutants vtc3-1 and vtc3-2 were previously isolated by a nitroblue tetrazolium-based genetic screen to identify AsA-deficient Arabidopsis mutants from an EMS mutagenized population (Col-0 background). The vtc3-1 and vtc3-2 mutants define VTC3 (Vitamin C 3) and were used to initially map this locus to a position close to VTC1 on chromosome 2, ~4 cM centromere distal from microsatellite marker nga168 (Conklin et al., 2000). Using a mapping population of approximately 2000 F₂ individuals derived from a cross between vtc3-1 and the wild-type Ler ecotype, the VTC locus was further narrowed genetically to a ~82 kb region between a microsatellite on BAC T7D17 (on chromosome 2, from 16986782 to 16987027), and InDel CER 459190 on BAC T20B5 (on chromosome 2 at 17068724; Fig. 1). This region contains 20 genes, none of which annotated as candidate AsA biosynthetic enzyme-encoding genes. To identify which of the 20 genes is VTC3, the coding region of each candidate in the AsA-deficient vtc3-1 and vtc3-2 mutants from the candidate AGI locus At2g40860. VTC3 was genetically mapped to a ~1.86 Mb genomic region. Fine-mapping placed VTC3 between a microsatellite (A) and InDel CER 459190 (B) within an ~82 kb region that contains 20 candidate genes (small black arrows). The mutants vtc3-1 and vtc3-2 were found to harbour single-base substitution mutations in At2g40860 (grey arrow).
complementation of the vtc3-1 and vtc3-2 alleles was undertaken. If At2g40860 is indeed VTC3, T-DNA insertion mutants in this locus should also have the AsA-deficient phenotype. Furthermore, this deficiency in the EMS mutant alleles should be relieved upon the transgenic introduction of a wild-type At2g40860 coding sequence. Salk 066988c and Salk 09574 T-DNA insertion mutants (Alonso et al., 2003) were confirmed to be homozygous for T-DNA insertions in exon 7 of At2g40860 (Fig. 3A). The two At2g40860 insertion mutant lines have an AsA deficiency similar to that of the two EMS-derived vtc3 mutants, confirming that a mutant allele of At2g40860 shares the same phenotype as the vtc3-1 and vtc3-2 mutants (Fig. 2B). Wild-type, vtc3-1, and vtc3-2 plants were transformed with a wild-type copy of the At2g40860 cDNA (U11316, Yamada et al., 2003) to test for transgenic complementation of vtc3 mutants by wild-type At2g40860. T1 hygromycin-resistant individuals were selected in each genetic background. Pooled hygromycin-resistant T2 plants were assayed for total AsA. Antibiotic resistant siblings were confirmed by PCR to contain the transgene. The At2g40860 cDNA (labelled ‘eVTC3’) complemented the genetic defect in both vtc3-1 and vtc3-2, as these transgenic lines are no longer AsA-deficient (Fig. 3C). Together, these results provide genetic evidence that the At2g40860 insertion mutant alleles vtc3-1 and vtc3-2 are allelic. Interestingly, expression of the At2g40860 cDNA from the 3SS promoter did not further elevate AsA levels in the transgenic wild-type plants.

**VTC3 is predicted to encode a novel highly conserved dual function protein kinase/protein phosphatase 2C**

VTC3 (At2g40860) is predicted to encode a 72.9 kD (658 amino acids) polypeptide with two conserved domains: an N-terminal catalytic domain of a protein kinase (aa 30–314, conserved domain cd00180; 9.44e-46) and a C-terminal Ser/Thr protein phosphatase 2C domain (aa 391–641, cd00143, 3.15e-6; Fig. 2). The putative VTC3 protein kinase domain is likely a Ser/Thr-specific protein kinase as it contains an invariant Ser/Thr protein kinase active site signature, ProSite PS00108 (145 IvHrDVKpaNVLL 157). The eukaryotic kinase superfamily members contain 12 conserved subdomains and share 12 invariant or nearly invariant residues within these subdomains (Hanks and Hunter, 1995). Of these 12 highly conserved residues, the VTC3 predicted kinase domain contains nine. Using the *Mus musculus* PKA-Cα residue numbering for reference, these include the equivalent of G52 K72, D166, N171, D184, G186, E208, D220, and G225. An alignment of VTC3, *Mus musculus* PKA-Cα, and *Homo sapiens* CDK2, including conserved kinase subdomains VII/VIII (residues 147–210 in VTC3), is shown in Fig. 2 with the highly conserved residues D166, N171, D184, G186, E208 noted. The region shown includes the conserved catalytic loop (HRDLKXXX) and the activation loop, defined as the region between and including the conserved DFG and APE motifs. In many kinases, phosphorylation (or autophosphorylation) of Tyr, Ser, and/or Thr residue(s) within the activation loop triggers a conformation change, allowing access of the protein substrate to the active site. It is therefore likely that a residue(s) within the activation loop of the VTC3 kinase domain is subject to phosphorylation. NetPhos (Blom et al., 1999) was used to predict if any residues within this loop are potential targets for phosphorylation. One serine residue (S188; score 0.995) was predicted as a likely site of phosphorylation but experimentation will be needed to confirmation this prediction. The G202E missense mutation in vtc3-1 is located within the activation loop (Fig. 2). Modelling of the wild-type and mutant kinase domain using SWISS-MODEL (http://swissmodel.expasy.org/) suggested that the missense (Fig. 2). An alignment of VTC3 (aa 423–449) with the human PP2C-α (P35813) and human PP2C-β (O75688), beta-Strand 4 (β4) and alpha-helix 1 (α1) domains is shown. Residues within β4 that are highly conserved within the most diverse members of the PP2C family (NCBI CCD cd00143) are in bold. The invariant metal-coordinating residues noted in the text are highlighted with carets (^). The location of the nonsense mutation (Q448X; *’) in the mutant vtc3-2 C-terminal end of α1 is shown.

**Fig. 2.** VTC3 (At2g40860) is predicted to encode a dual function polypeptide with an N-terminal catalytic ST protein kinase (aa 30–314) domain and a C-terminal ST protein phosphatase 2C domain (aa 391–641). An alignment of the predicted amino acid residues of VTC3 (147–210) with the *Homo sapiens* CDK2 (CAA43807) and *M. musculus* PKA-Cα (NP_032880) is shown. Bolded lettering in the protein kinase domain indicates residues conserved in at least two of the three polypeptides. The residues that comprise the conserved HRD catalytic loop (dashed line), the activation loop (solid line, with predicted sites of phosphorylation underlined), and five of the invariant or nearly invariant eukaryotic kinase domain residues (*') are noted. The missense mutation (G202E) in the predicted polypeptide encoded by vtc3-1 is shown. An alignment of VTC3 (aa 423–449) with the human PP2C-α (P35813) and human PP2C-β (O75688), beta-Strand 4 (β4) and alpha-helix 1 (α1) domains is shown. Residues within β4 that are highly conserved within the most diverse members of the PP2C family (NCBI CCD cd00143) are in bold. The invariant metal-coordinating residues noted in the text are highlighted with carets (^). The location of the nonsense mutation (Q448X; *’) in the mutant vtc3-2 C-terminal end of α1 is shown.
mutation in vtc3-1 significantly alters the conformation of the activation loop (data not shown). Furthermore, the equivalent glycine in cAMP-dependent protein kinase (G200) has been shown to be involved in substrate binding (Moore et al., 2003) as has the equivalent glycine (G206) in the tomato PTO kinase (Wu et al., 2004). In a survey of 100 diverse members of the NCBI CCD cd00180 protein kinase family, the most common residue at this position (54/100) is a glycine. None of these diverse protein kinases contains the acidic glutamic acid residue. Therefore it is predicted that the G202E mutation in vtc3-1 negatively impacts the function of the VTC3 kinase domain.

The C-terminal portion of VTC3 contains a predicted type 2C protein phosphatase domain. Protein phosphatases are classified into one of three main families and the PPM/PP2C family of Ser/Thr phosphatases is thought to be evolutionarily unrelated to the other protein phosphatases (PPP, FCP/SCP) and is distinguished by a requirement for Mn\(^{2+}\) or Mg\(^{2+}\) and insensitivity to certain phosphatase inhibitors. Unlike other Ser/Thr phosphatases, the PP2Cs are thought to generally lack association with a regulatory subunit. The human genome contains at least 16 different PP2C genes. In contrast, there are 80 PP2C genes in Arabidopsis thaliana and 78 in Oryza sativa (Xue et al., 2008). Several of these PP2Cs are quite closely related and are likely the result of recent duplication events. However, within 13 different PP2C subfamilies, At2g40860 (VTC3) and its rice homologue reside alone in subgroup G (Xue et al., 2008). Similarly, an older analysis of Arabidopsis PP2C genes also classified At2g40860 in its own clade, as PP2C-1D (Kerk et al., 2002). PP2Cs contain six invariant metal binding residues that coordinate binding of a Mn\(^{2+}\) or Mg\(^{2+}\) ion in the catalytic site. The predicted VTC3 PP2C domain contains all six of these residues (three are shown in Fig. 2). Eukaryotic PP2Cs contain 10 invariant residues (Das et al., 1996) scattered across 11 conserved motifs (Bork et al., 1996) and VTC3 also shares these conserved residues and motifs (data not shown). Domain architecture prediction using SWISS-MODEL predicts the presence of the six \(\alpha\)-helices and eleven \(\beta\)-strands that are hallmarks of the PP2C family (Das et al., 1996). Two of these (\(\beta4\) and \(\alpha1\)) are within VTC3 residues 423–449, shown aligned with human PP2C-\(\alpha\) and PP2C-\(\beta\) (Fig. 2). Given this conservation with other known PP2Cs, it is likely that the PP2C domain encoded by the wild-type allele of VTC3 is functional. In contrast, the Q448X nonsense mutation in the vtc3-2 allele is predicted to prematurely terminate the PP2C domain C-terminal at the C-terminal end of the first \(\alpha\)-helix (\(\alpha1\); Fig. 2), resulting in a probable non-functional protein product missing 211 amino acids from the C-terminus of wild-type VTC3.

![Fig. 3.](https://academic.oup.com/jxb/article-abstract/64/10/2793/542524)

Fig. 3. The AsA-deficient phenotype of At2g40860 insertion mutants and transgenic complementation of the AsA deficiency of vtc3-1 and vtc3-2 with a wild-type At2g40860 cDNA confirm the identity of the VTC3 locus. (A) Insertion lines Salk 095743 and Salk 066988C were confirmed to harbour insertions in exon 7 of At2g40860. (B) Total AsA was assayed in 2-week-old seedlings from the wild type, the insertion mutant lines, and the vtc3 EMS-generated mutants. (C) Wild type, vtc3-1, and vtc3-2 were transformed with At2g40860 cDNA driven by the CaMV 35S promoter. Total AsA was assayed in pooled hygromycin-resistant T2 progeny. Designations L1 and L2 indicate independent transgenic lines. For all AsA assays, the average total AsA expressed as \(\mu\)mol g\(^{-1}\) freshweight (FWT) is shown for each line (three assays from replicate extracts). Bars indicate SD.
VTC3/AT2G40860 is highly conserved amongst the Viridiplantae

VTC3 orthologues exist across the Viridiplantae from green algae such as *Chlamydomonas* to the eudicots, dicots, bryophytes, and lycophytes (Fig. 4). Maximum percentage identity of these orthologues with VTC3 is quite high, for example 70% identity with *Populus trichocarpa* and 55% with *Selaginella moellendorfii*. A probable conifer (*Picea glauca*) homologue with 61% maximum identity with VTC3 was found only after manual translation of an mRNA (NCBI GI: 270139925) derived from several expressed sequence tags. VTC3 orthologues are noticeably absent outside this major phylogenetic grouping. Although the cyanobacteria (e.g. *Trichodesmium erythraeum*) also harbour a polypeptide annotated as containing a N-terminal protein kinase domain and a C-terminal PP2C domain, this protein (unlike the putative orthologues) has limited similarity to VTC3 and only within the PP2C domain (24% amino acid identity over 238 aa). The presence of VTC3 orthologues amongst the liverworts, hornworts, and ferns could not be established, although protein database information on these groups is somewhat limited.

**VTC3 appears to be plastid localized**

Given the probable regulatory role of VTC3, it is of interest to determine the subcellular locale of this polypeptide. Various localization predictor tools place VTC3 in either the plastid (IPSORT; Multiloc, Predotar, WoLFPSORT) or the cytosol (SubLoc, TargetP). To experimentally determine the location of VTC3, this study generated plants that expressed a YFP::VTC3 fusion protein and cloned a full-length *VTC3* cDNA (pUNI G11316) into the *Agrobacterium tumefaciens* transformation vector pEarleygate 104 (Earley et al., 2006) to generate an N-terminal YFP fusion. The subcellular location of VTC3 inside transformed plant cells was then visualized by YFP fluorescence as shown in Fig. 5. Mature leaf tissue from 3-week-old glufosinate-resistant T1 plants was imaged using fluorescence confocal laser scanning microscopy. YFP was absent from the control plant, but fluoresced in the same location as the chlorophyll in the leaf tissue from the N-terminal YFP::VTC3 transgenic plant. Therefore, it is conclude that VTC3 is localized to the plastid at least under the growth conditions utilized in this study.

VTC3 is predicted to contain a transmembrane helix from 276–292 aa (TMpred, score 888; Hoffmann and Stoffel, 1993) with the N-terminus of VTC3 located outside of the membrane. This 17-aa domain is located just C-terminal to the active site of the kinase domain (Fig. 2), putatively placing the kinase domain on the outside surface of a membrane.

**The redox status of AsA is not altered in the vtc3-1 mutant**

A deficiency in AsA could result from either a defect in synthesis or a defect in turnover. If VTC3 is involved in the regulation of AsA recycling via dehydroascorbate and/or monodehydroascorbate reductases, a deficiency in VTC3 is predicted to lead to a reduction in the ratio of reduced to total AsA. To test this, wild-type and *vtc3-1* plants were grown under standard conditions and the AsA redox status was determined. There was no significant difference in the AsA redox status in these two genotypes (wild-type reduced AsA/total AsA = 0.85 ± 0.02; *vtc3-1* reduced AsA/total AsA = 0.80 ± 0.08), suggesting that VTC3 is likely not involved in maintenance of AsA levels via redox recycling.

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**Fig. 4.** Close orthologues of VTC3 exist across the Viridiplantae and are apparently absent outside this large group. MUSCLE (Edgar, 2004) was utilized to generate an amino acid alignment between *Arabidopsis thaliana* VTC3 and apparent VTC3 orthologues. The two cyanobacterium polypeptides were chosen as outgroups. The phylogram was generated with the neighbour-joining method (BioNJ; 100 bootstrap replications; Gascuel, 1997) and drawn with TreeDyn (Chevenet et al., 2006). Branches having <0.5 bootstrap support have been melded into single larger branches.
VTC3, a protein kinase::phosphatase involved in AsA pool size

As the last step in the SW AsA biosynthetic pathway (conversion of l-galactono-1,4-lactone to AsA, catalysed by l-galactono-1,4-lactone dehydrogenase) is regulated, it is possible that VTC3 plays a role in AsA synthesis at this point in the pathway. Feeding of this substrate to detached leaves of various plant species results in its conversion to AsA. Soil-grown wild-type and vtc3 insertion mutant (Salk 066988) were preincubated in the dark for 24 h, and then detached whole rosettes were fed through the transpiration stream with 10 mM l-galactono-1,4-lactone for 24 h in the light. Samples were taken at 0, 6, and 24 h for assay of total AsA. The average total AsA expressed as μmol/g freshweight (FWT) is shown for each sample (three assays from replicate extracts). Bars indicate SD.

vtc3 mutants are defective in heat- and light-induced elevation of AsA

As mentioned previously, an increase in the AsA pool in response to light is well documented. Plants also respond to heat in a similar fashion (Hoeberichts et al., 2008). To determine if VTC3 is involved in this regulation, wild-type and various vtc3 mutant genotypes were exposed to different environmental conditions, and the amount of AsA was assayed from plants sampled at several time points.

To assay the response of the vtc3 mutants to continuous light at an elevated temperature, total AsA in the wild type and three vtc3 mutants (vtc3-1, vtc3-2, Salk 06688C ‘vtc3 KO’) was determined after transfer from 16 °C (12/12 light/dark cycle) and 30 °C (continuous light). Samples were taken for analysis on days 0, 2, and 4 of the high temperature/continuous light exposure. The results (Fig. 7A) suggest that relative to the wild type, all the vtc3 mutants were defective in the ability to respond significantly to high temperature/continuous light stress through upregulation of AsA. By day 4, the level of AsA in the wild type increased ~3.2-times whereas the corresponding response of the mutants was significantly less dramatic (vtc3-1, ~2.3-times; vtc3-2, ~1.2-times; vtc3 KO, ~1.2-times).

The possible role of VTC3 in the light regulation of AsA was also evaluated. Total AsA levels were assayed in wild-type, vtc3-1, and vtc3-knockout (Salk 06688C) seedlings exposed to light following an initial dark incubation for 24 h. These mutants were somewhat defective in the light-regulated elevation of AsA relative to the wild type (Fig. 7B). Over the course of the 48-h light exposure, AsA levels steadily rose in the wild type ~1.6-times relative to the initial 0 h time point, in contrast to the fairly steady state level of AsA in the mutants.

L-Galactono-1,4-lactone conversion to AsA is unchanged in a vtc3 insertion mutant

As the last step in the SW AsA biosynthetic pathway (conversion of l-galactono-1,4-lactone to AsA, catalysed by l-galactono-1,4-lactone dehydrogenase) is regulated, it is possible that VTC3 plays a role in AsA synthesis at this point in the pathway. Feeding of this substrate to detached leaves of various plant species results in its conversion to AsA. Soil-grown wild-type and vtc3 insertion mutant (Salk 066988c) were preincubated in the dark for 24 h, and then detached whole rosettes were fed through the transpiration stream with 10 mM l-galactono-1,4-lactone for 24 h in the light. Samples were taken at 0, 6, and 24 h for assay of total AsA. The average total AsA expressed as μmol/g freshweight (FWT) is shown for each sample (three assays from replicate extracts). Bars indicate SD.
over time. The light levels used in this experiment were moderate (~120 PPFD) and it is possible that conducting the experiment at higher light levels would serve to accentuate this observed difference between the light response of the wild type and the vtc3 mutants.

Discussion

VTC3 is predicted to encode a highly novel dual function Ser/Thr protein kinase::protein phosphatase 2C. This gene is highly conserved across the Viridiplantae/Chloroplastida. No close orthologues could be found outside this major phylogenetic grouping including within the Glaucophyta. Therefore, it is likely that this is an ancient gene, present in the most common recent ancestor of the Viridiplantae/Chloroplastida (green algae, land plants), arising after its split with the ancestor to the present-day Rhodophyta (red algae). VTC3 could have emerged concomitant with emergence of the α-mannose/β-galactose pathway for the synthesis of AsA.

The AsA-deficient vtc3 mutants are defective in their ability to elevate AsA in response to both heat and light (Fig. 7). In contrast, Conklin et al. (2000) showed that vtc3-1 is unaffected in its ability to proportionally increase the AsA pool in specific tissues (green siliques, inflorescence). Therefore, it is proposed that the VTC3 dual protein kinase/protein phosphatase is involved in signal transduction, adjusting AsA levels in response to changing environmental conditions. In plants, the regulation of the AsA pool in leaf tissue is not well understood but likely occurs both at the level of recycling/turnover and biosynthesis. As the redox status of AsA is unaltered in the vtc3-1 mutant relative to the wild type, it is unlikely that VTC3 affects AsA recycling.

As detailed in the introduction, component(s) of the SW pathway responds to light at the transcriptional level. Investigations are underway as to whether or not VTC3 acts at the transcriptional level to exert control over AsA metabolism. Preliminary quantitative PCR data suggest that biosynthetic enzyme transcript levels are unchanged in the vtc3 mutants relative to the wild type (data not shown). This has been corroborated by whole transcriptome shotgun sequencing (N. Smirnoff and M. Page, personal communication). Data mining of existing transcript profiling data indicates that the expression of VTC3 itself is fairly constitutive over different developmental stages and in response to varied abiotic stresses and phytohormone treatments (Supplementary Fig. S1). The exception being that VTC3 transcripts appear to be highly abundant in the root of the torpedo stage embryo (Casson et al., 2005).

If VTC3 controls AsA biosynthesis, this control could be post-transcriptional. With two signal transduction domains (protein kinase, protein phosphatase) within one novel polypeptide, a variety of possible mechanisms can be invoked. Both the kinase and the PP2C domains could act on another protein target, with either phosphorylation or dephosphorylation dominant, depending on the environmental condition. Alternatively, the kinase domain could be a substrate for the PP2C domain or vice versa. In any case, it is proposed

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**Fig. 7.** Arabidopsis vtc3 mutants are ineffective at elevation of total AsA in response to heat and to light. (A) Soil-grown 2-week-old Arabidopsis (Col-0 wild-type and four vtc3 mutant lines) were exposed to 30 °C growth conditions under continuous illumination for 4 days. Leaf tissue was harvested for analysis of total AsA (n = 3) at days 0, 2, and 4. (B) Soil-grown 2-week-old Arabidopsis (Col-0 wild-type, vtc3-1, Salk 066988c) were subjected to a 24-h dark treatment prior to exposure to continuous light. Tissue was harvested for total AsA analysis (n = 4) at 0, 8, 24, and 48 h. Control plants remained in the dark for the duration of the experiment. Within-genotype one-way ANOVA with Tukey post hoc analysis was performed; the same lower case letter above two means indicates no statistically significant difference between those means. Bars indicate SD.
that VTC3 could affect the activity of an AsA biosynthetic enzyme(s) through a signal transduction cascade that transduces an external stimulus to a response, in this case an elevation of the AsA pool.

As most protein kinases operate in a positive capacity, one could speculate that the VTC3 kinase domain activates AsA biosynthesis. The vt3-1 mutant contains a mutation that is predicted to alter the conformation of the kinase activation loop (T-loop) and is AsA deficient, lending support for this hypothesis. This activation loop could very well be subject to autophosphorylation. Although there is no proteomics-based experimental evidence that the activation loop of the VTC3 kinase is phosphorylated, phosphorylation prediction programs indicate that S188 (score 0.995, NetPhos 2.0, Blom et al., 1999) and T192 (score 0.785, PhosPhAT 4.0, Durek et al., 2010) within the activation loop are likely sites of phosphorylation (Fig. 2). An active kinase domain of VTC3 could directly or indirectly activate a biosynthetic enzyme via phosphorylation.

Alternatively or in addition, a substrate of the kinase domain could be the PP2C phosphatase domain. The VTC3 PP2C domain is a member of a large family of PP2C protein phosphatases (as detailed above and reviewed by Fuchs et al., 2013). Mass spectrometry indicates that in suspension cells, the T466 within the PP2C domain of Arabidopsis VTC3 is phosphorylated in vivo (Sugiyama et al., 2008). The threonine at this position is conserved within the eudicot and monocot VTC3 homologues as well as in the lycophyte (Fig. 4) and falls within the second PP2C α-helical domain (Das et al., 1996). This phosphorylation could have biological significance, as there is abundant evidence for phosphorylation state regulation of PP2C activity (Peraldi et al., 1994; Allemand et al., 2007; Awano et al., 2008; Sajid et al., 2011).

What is the role of the VTC3 PP2C domain in regulation of AsA levels? The vt3-2 mutant has a nonsense mutation early in the predicted PP2C domain, therefore it likely that this mutant has defective phosphatase activity. As this mutant has the same phenotype (AsA deficiency) as the vt3-1 and knockout mutants, it is plausible that (along with the kinase domain) this PP2C domain also has a positive role in the control of AsA levels. Possibly, a substrate of the VTC3-PP2C is the kinase domain. A textbook example of a kinase that is activated by dephosphorylation is the activation of human kinase CDC2 by the phosphatase CDC25. Although unusual, there are cases in which PP2Cs are active in a positive role. For example, human PP2Ca positively regulates insulin sensitivity via direct activation of kinase P13K (Yoshizaki et al., 2004). In an alternative scenario, PP2C could be acting in a negative capacity with the truncated polypeptide produced from the vt3-2 allele being either degraded or folded aberrantly such that the kinase domain is also non-functional.

The data suggest that VTC3 is localized to the plastid in light-grown seedlings and, given the presence of a possible transmembrane-spanning helix, may be anchored to the surface of a plastid membrane. Several proteomics-based studies have catalogued plastid-localized polypeptides and there is no evidence from these studies that VTC3 is indeed located in the plastid. Given the YFP localization results and the apparent absence of a plastid transit peptide, it is speculated that VTC3 could be anchored (perhaps loosely) to the outer membrane of the plastid perhaps enabling it to transduce plastid-sourced signals.

In summary, the unique Viridiplantae-specific VTC3 may be a dual function signal transduction protein, with perhaps the PP2C domain acting as a sensor relay that perceives a change in the environment and the covalently tethered kinase domain acting as a sensor responder to influence the cellular AsA level. This study group is currently investigating VTC3-interacting proteins to identify possible protein substrate(s) of VTC3 and is constructing transgenic Arabidopsis that overexpress either the kinase domain or the PP2C domain. In addition, recombinant VTC3 are being generated to verify the functional activity of the two domains. Such experiments will begin to tease apart the role of this unique protein in plant AsA biosynthesis.

Supplementary material

Supplementary data are available at JXB online.
Supplementary Table S1. Primers used to map the VTC3 locus.
Supplementary Table S2. Primers used to amplify and sequence the At2g40860 locus.
Supplementary Table S3. Primers used to genotype transgenic lines.
Supplementary Fig. S1. Compilation of existing expression data on VTC3 (At2g40860) mined from expression profiling experiments reported in the public Arabidopsis eFP Browser (Winter et al. 2007).

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