Antioxidant status, peroxidase activity, and PR protein transcript levels in ascorbate-deficient Arabidopsis thaliana vtc mutants

Louise Colville and Nicholas Smirnoff*

School of Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK

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Abstract

Ascorbate is the most abundant small molecule antioxidant in plants and is proposed to function, along with other members of an antioxidant network, in controlling reactive oxygen species. A biochemical and molecular characterization of four ascorbate-deficient (vtc) Arabidopsis thaliana mutants has been carried out to determine if ascorbate deficiency is compensated by changes in the other major antioxidants. Seedlings grown in vitro were used to minimize stress and longer term developmental differences. Comparison was made with the low glutathione cad2 mutant and vtc2-1 treated with D,L-buthionine-[S,R]-sulphoximine to cause combined ascorbate and glutathione deficiency. The pool sizes and oxidation state of ascorbate and glutathione were not altered by deficiency of the other. α-Tocopherol and activities of monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, and catalase were little affected. Ascorbate peroxidase activity was higher in vtc1, vtc2-1, and vtc2-2. Isonically bound cell wall peroxidase activity was increased in vtc1, vtc2-1, and vtc4. Supplementation with ascorbate increased cell wall peroxidase activity. 2,6-Dichlorobenzonitrile, an inhibitor of cellulose synthesis, increased cell wall peroxidase activity in the wild type and vtc1. The transcript level of an endochitinase, PR1, and PR2, but not GST6, was increased in vtc1, vtc2-1, and vtc2-2. Endochitinase transcript levels increased after ascorbate, paraquat, salicylic acid, and UV-C treatment, PR1 after salicylic acid treatment, and PR2 after paraquat and UV-C treatment. Camalexin was higher in vtc1 and the vtc2 alleles. Induction of PR genes, cell wall peroxidase activity, and camalexin in vtc1, vtc2-1, and vtc2-2 suggests that the mutants are affected in pathogen response signalling pathways.

Key words: cad2, camalexin, glutathione, hydrogen peroxide, oxidative stress, salicylic acid.

Introduction

The plant antioxidant system consists of a number of enzymes and low molecular weight compounds. This network is important both for controlling excessive reactive oxygen species (ROS) production during stress and in maintaining the correct levels of ROS for growth and signalling (Mittler et al., 2004). For example, ROS are involved in polarized cell growth (Foreman et al., 2003; Jones et al., 2007; Potocky et al., 2007), abscisic acid (ABA) signalling (Kwak et al., 2006), and pathogen responses (Mullineaux and Rausch, 2005). The two major soluble small molecule antioxidants are ascorbate and glutathione (GSH; γ-glutamylcysteineglycine). Ascorbate is capable of reducing superoxide, singlet oxygen, and hydroxyl radicals directly, and is also a substrate for ascorbate peroxidase (APX) which catalyses the conversion of hydrogen peroxide to water. GSH represents the most abundant non-protein thiol in plants. In addition to its role as an antioxidant, GSH is involved in sulphur metabolism and detoxification of xenobiotics by glutathione Stransferases, and is a precursor of phytochelatins, which are important in the control of metal concentrations within cells (Cobbett,
Ascorbate and GSH participate in the ascorbate–GSH cycle, through which oxidized ascorbate (dehydroascorbate) produced by hydrogen peroxide reduction is reduced by GSH-dependent dehydroascorbate reductase (DHAR), and the resulting GSH disulphide (GSSG) is reduced by glutathione reductase (GR) using NADPH as an electron donor (Noctor and Foyer, 1998). Mutants with altered levels of antioxidants have been invaluable in investigations into the roles of antioxidants in plants. Seven ascorbate-deficient vtc mutants have been identified (Conklin et al., 2000) and represent four different VTC loci. Two of the vtc mutants (vtc1-1 and vtc2-1) were identified by their increased sensitivity to ozone. VTC1 encodes GDP-mannose pyrophosphorylase (Conklin et al., 1999) and VTC4 encodes L-galactose 1-P phosphatase (Conklin et al., 2006), which are both enzymes in the proposed GDP-mannose pathway of ascorbate biosynthesis (Wheeler et al., 1998). VTC2 encodes GDP-L-galactose phosphorlase, also a biosynthetic enzyme (Dowdle et al., 2007). The identity and function of VTC3 are unknown. The ascorbate levels in the vtc mutants range from 50% of wild-type levels in vtc2-3, vtc3, and vtc4, to 25–30% in vtc1-1, vtc2-2, vtc2-1, and vtc2-2. Characterization of vtc1-1 and vtc2-1 has revealed that both mutants have a slower growth rate (Conklin et al., 2000; Pastori et al., 2003; Pavet et al., 2005), but there is no effect on photosynthesis rate or hydrogen peroxide levels (Veljovic-Jovanovic et al., 2001; Muller-Moule et al., 2004) in plants grown under moderate light intensity. Recently, double mutant Arabidopsis thaliana plants unable to synthesize ascorbate due to lack of functional VTC2 and VTC5 (a homologue of VTC2) have been produced. These are unable to grow unless supplemented with ascorbate, showing that it is essential for seedling growth (Dowdle et al., 2007). The vtc1 and vtc2 mutants have been most widely studied in relation to their stress responses, while there is little information on vtc3 and vtc4. The mutants show a varied response to ozone, with vtc1-1, vtc1-2, and vtc2-1 showing extreme sensitivity to ozone, whilst the other vtc mutants were only slightly more sensitive to ozone than wild-type plants (Conklin et al., 2000). Therefore, ozone sensitivity appears not to be entirely dependent on ascorbate levels. vtc1 is more sensitive to UV-B (Conklin et al., 1996; Gao and Zhang, 2008) and UV-C radiation (Filkowski et al., 2004). Since ascorbate is a cofactor for violaxanthin de-epoxidase, vtc2 has reduced ability to synthesise zeaxanthin under high light and is more susceptible to photooxidative stress (Muller-Moule et al., 2004). All four mutants are more salt sensitive (Smirnoff, 2000; Huang et al., 2005). vtc1 and vtc2 have reduced basal thermotolerance (Larkindale et al., 2005) and vtc2-1 has greater thermoduced photon emission, indicating increased lipid peroxidation at high temperature (Havaux, 2003). Therefore, there is strong evidence that low ascorbate both reduces growth and increases susceptibility to a range of stresses.

Pastori et al. (2003) performed transcriptomic analysis of the vtc1-1 mutant compared with the wild type using microarrays. They identified 171 genes that were differentially expressed in vtc1-1; of these, 12.9% were genes involved in cell defence. In particular, pathogenesis-related (PR) proteins showed a relatively high level of induction. ABA concentration was 60% higher in vtc1-1 than in the wild type, so ABA signalling may provide a link between ascorbate levels and PR protein transcript level. vtc1-1 and vtc2-1 both have increased resistance to infection by virulent pathogens (Barth et al., 2004; Pavet et al., 2005). In contrast to the results obtained by Pastori et al. (2003), Barth et al. (2004) found that PR proteins were not more highly expressed in vtc1-1 than in the wild type, but were induced more strongly by pathogen infection, due to higher levels of salicylic acid (SA). Despite the fact that vtc1-1 has greatly reduced ascorbate levels, there appear to be no adverse phenotypic effects resulting from increased oxidative stress. There is also no consistent evidence for an increase in the levels of other antioxidants such as GSH or tocopherol, or antioxidant enzymes. This suggests that under normal conditions the low intracellular level of ascorbate is sufficient for scavenging ROS. Studies into the effects of high light on vtc2-1 have shown that ascorbate deficiency resulted in a reduced acclimation to high light with increased signs of oxidative stress, such as lipid peroxidation and bleaching. In high light, the vtc2-1 mutant had 30% higher levels of GSH than the wild type. Therefore, there appears to be a compensatory mechanism for reduced levels of ascorbate under environmental stress conditions (Muller-Moule et al., 2003). Another feature of vtc1 and ascorbate-deficient plants expressing antisense L-galactose dehydrogenase is increased activity of peroxidase other than APX (Veljovic-Jovanovic et al., 2001; Gatzek et al., 2002).

The GSH-deficient Arabidopsis thaliana mutant, cad2-1, has 15–30% of the GSH concentration compared with wild-type plants. It is deficient in γ-glutamylcysteine synthetase, the first enzyme in the GSH biosynthesis pathway. The mutant is also deficient in phytochelatins and has increased sensitivity to cadmium. In the absence of cadmium, cad2-1 does not show any phenotypic effects of reduced GSH levels; therefore, as in the ascorbate-deficient mutants, the residual amount of GSH is sufficient for maintaining normal cellular functions (Cobbett et al., 1998). However, more severe mutations or knockouts in γ-glutamylcysteine synthetase result in lack of root growth or embryo development (Vernoux et al., 2000; Cairns et al., 2006). Like ascorbate, GSH has also been implicated in redox processes associated with responses to pathogens (Ball et al., 2004). However, the 70% reduction in GSH concentration of cad2 does not affect its susceptibility to pathogens (May et al., 1996).
The purpose of this investigation was first to assess the status of the antioxidant system in a range of ascorbate-deficient *A. thaliana* vtc mutants to determine if ascorbate deficiency results in changes in the other major antioxidants related to the ascorbate–GSH cycle. The *cad2* mutant and treatment with DL-buthionine-[S,R]-sulphoximine (BSO) to inhibit GSH biosynthesis (May and Leaver, 1993) were used to determine the effect of a combination of low ascorbate and GSH. Secondly, since the results from a number of different experiments suggest that ascorbate-deficient mutants have increased SA, an increased transcript level of genes encoding PR proteins, and increased resistance to virulent pathogens, peroxidase activity, PR gene transcript level, and accumulation of the phytoalexin camalexin were found in the vtc mutants, most consistently in vtc1 and vtc2-1. Attempts to reverse high cell wall peroxidase activity and PR protein transcript level in vtc mutants by exogenous ascorbate instead caused a large increase in both. The results are discussed in relation to the role of ascorbate in pathogen defence responses.

### Materials and methods

#### Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia-0, vtc mutants (Conklin et al., 2000), and cad2-1 (Cobbett et al., 1998) were grown *in vitro* on Gilroy medium containing 0.38 mM sucrose and 0.1% Phytagel (Jones et al., 2002) in growth cabinets with a 12 h light (20 °C)/12 h dark (15 °C) cycle. The photosynthetic photon flux density was 180 μmol m⁻² s⁻¹. For all assays and total RNA extraction, 100 mg fresh weight of the aerial parts of whole 14-day-old seedlings were harvested.

#### Stress and ascorbate treatments

Aqueous solutions (5 ml) of 10 mM ascorbate, 1 mM SA, or 10 mM paraquat were applied to the Petri dishes on which the seedlings were growing, and incubated for 24 h under normal growth conditions. For the UV-C treatment, seedlings growing on Petri dishes were exposed to UV-C radiation in a Strатаген UV Crosslinker for 30 min, which supplied a dose of 44 kJ m⁻² s⁻¹ followed by 24 h under normal growth conditions. Wild-type and vtc2-1 plants were also grown in 0.5 mM BSO to deplete the GSH pool.

#### Measurement of ascorbate, glutathione, and α-tocopherol concentration

For determination of ascorbate and GSH concentration, 100 mg of whole seedlings was harvested and immediately frozen in liquid nitrogen. Samples were homogenized using a pestle and mortar with liquid nitrogen, followed by the addition of 0.5 ml of 0.1 N HCl containing 1 mM Na₂EDTA. The homogenate was centrifuged at 13 000 g for 2 min.

Ascorbate was measured using Fe³⁺ reduction and Fe²⁺ detection with bipyridyl (Kampfenkel et al., 1995) scaled down for microtitre plates. Tests using ascorbate oxidase to remove ascorbate from the extracts showed that *A. thaliana* plants grown under the conditions used in these experiments do not contain significant concentrations of other compounds that reduce Fe³⁺. Glutathione was measured with a GR-linked assay (Baker et al., 1990).

Tocopherol was extracted from 100 mg of tissue by homogenization in 450 μl of methanol: dichloromethane (2:1, v/v) containing 1 mg ml⁻¹ butylated hydroxytoluene (BHT, Sigma). A 300 μl aliquot of high-performance liquid chromatography (HPLC) grade water and 150 μl of dichloromethane (Fisher) were added to the homogenate and vortexed for 30 s. The samples were then centrifuged at 3700 g for 10 min. A 200 μl aliquot of the organic phase was evaporated to dryness under nitrogen, and resuspended in 100 μl of 3% (v/v) dichloromethane in methanol. Then 20 μl was analysed by HPLC using a C18 column (4.6 μm×250 μm×5 μm) (Phenomenex) and fluorescence detection (λex 290 nm and λem 325 nm). Isocratic elution was performed with a mobile phase consisting of 3% (v/v) dichloromethane in methanol, and a flow rate of 0.5 ml min⁻¹. α-Tocopherol was quantified by comparison with a standard curve produced using known concentrations of an α-tocopherol standard (Sigma).

#### Enzyme extraction and assays

Enzymes were extracted by homogenizing leaf tissue in ice-cold extraction buffer (1 ml per 100 mg fresh weight), centrifuged at 13 000 g for 2 min, and kept on ice until assayed. The buffer used for the extraction of monodehydroascorbate reductase (MDHAR), DHAR, and GR consisted of 100 mM tricine, 1 mM Na₂EDTA, 5% (v/v) polyvinylpyrrolidone (PVP)-40, 20% (v/v) glycerol and 2 mM dithiothreitol (DTT), which was added just before use. APX was extracted in 50 mM potassium phosphate (pH 7.0), 1 mM Na₂EDTA, 0.1% (v/v) Triton X-100, and 1 mM sodium ascorbate, which was added just before use.

The MDHAR assay comprised 120 μl of 50 mM TRIS buffer (pH 7.6) containing 1 mM Na₂EDTA, 10 μl of 4.29 mM NADH, 10 μl of 2.5 mM sodium ascorbate, and 40 μl of extract. A 20 μl aliquot of ascorbate oxidase (5.6 U ml⁻¹, from *Curcubitac species*, Sigma) was added and the oxidation of NADH (Sigma) was followed at 340 nm (ε=6.22 mM⁻¹ cm⁻¹) in a microtitre plate reader (Smirnoff and Colombé, 1988).

The assay for GR consisted of 110 μl of 100 mM tricine (pH 7.6), 20 μl of 3.24 mM NADPH, 50 μl of extract, and 20 μl of 16 mM oxidized glutathione (GSSG). The oxidation of NADPH was followed at 340 nm (ε=6.22 mM⁻¹ cm⁻¹) in a microtitre plate reader (Smirnoff and Colombé, 1988).

The APX assay consisted of 910 μl of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM Na₂EDTA, 20 μl of 25 mM sodium ascorbate, 50 μl of extract, and 20 μl of 5 mM hydrogen peroxide in a total volume of 1 ml. The oxidation of ascorbate was followed at 280 nm (ε=7.83 mM⁻¹ cm⁻¹) (Smirnoff and Colombé, 1988).

GSH-dependent DHAR activity (Nakano and Asada, 1980) was measured in a reaction mixture containing 860 μl of 50 mM potassium phosphate buffer (pH 7.0) with 0.1 mM Na₂EDTA, 20 μl of 125 mM GSH, 20 μl of 10 mM dehydroascorbate, and 100 μl of extract. Ascorbate formation was followed at 280 nm (ε=7.83 mM⁻¹ cm⁻¹).

For catalase, leaves (100 mg) were extracted by homogenization in 400 μl of 50 mM KH₂PO₄ (pH 7.0) containing 0.1% (v/v) Triton X-100 and 1% (v/v) PVP-40 followed by centrifugation at 13 000 g for 15 min. The assay consisted of 100 μl of extract, 890 μl of
50 mM KH2PO4, and 10 μl of 10 mM H2O2, and the decomposition of H2O2 was followed at 240 nm (c=0.04 mM−1 cm−1).

Peroxidase activity was measured in the same extracts as those used for the MDHAR, DHAR, and GR assays. The assay consisted of 950 μl of assay buffer (50 mM KH2PO4, pH 7.0 and 1 mM Na2EDTA), 20 μl of 1 M pyrogallol, and 10 μl of extract in a total volume of 1 ml. The reaction was initiated by the addition of 20 μl of 150 mM H2O2, and the oxidation of pyrogallol was followed at 430 nm using an extinction coefficient of 2.47 mM−1 cm−1 (Veljovic-Jovanovic et al., 2001).

Total RNA extraction and qPCR
Qiagen Rneasy Mini Plant kits were used to extract total RNA. RNA was quantified spectrophotometrically and treated with RNase-free DNase (Promega). Reverse transcription was performed on 1 μg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen) and oligo(dT)15 (Sigma) primers, followed by treatment with RNase H (Ambion). The resulting cDNA was used for qPCR, which was performed using a BioRad iCycler and ABsolute QPCR SYBR Green Fluorescein mix (ABGene).

The following primers were designed using Beacon Designer (BioRad), EF1α (At5g60930), 5'-AACCTCTCTTGGAGGCTTTGGAG-3' and 5’-ACGGTTCCAATACCACCAATC-3’; endochitinase (At2g43570), 5’-TGGCAGACAGAAAACACAGAG-3’ and 5’- TTGGCAGACAGAACCCAGTTG-3’; GST6 (At2g37730), 5’-GGTCTGGCGCTGGATTC-3’ and 5’-CTTAGGGCGAG-AGTCAAAG-3’; PR1 (At2g14610), 5’-GCTAGCGAGAACTAAGAG-3’ and 5’-GGGACGCCAGACAAG-3’; PR2 (At3g57260), 5’-CTTAGGCTTCACCAATGTTG-3’ and 5’-TCCTGGTAGATCTCCGAGTTG-3’. Ct values for each gene were determined for three replicates of each treatment. The transcript level relative to the EF1α control gene was calculated by the comparative Ct method: 10^(-ΔΔCt)=100%, where ΔCt is the difference in Ct between EF1α and the target gene (Li et al., 2005).

Detection of camalexin by thin-layer chromatography (TLC)
TLC was used to identify and give a qualitative estimate of the levels of camalexin present in the vtc and cad2 mutants. To aid the identification of camalexin, a silver nitrate treatment was used to induce camalexin synthesis in the seedlings, so that treated and untreated samples could be compared. The silver nitrate treatment was applied by spraying 2-week-old seedlings growing in Petri dishes with 10 mM silver nitrate (BDH) in 0.02% (v/v) Silwet (Zhao and Last, 1996). The extraction procedure and TLC analysis were performed according to a method described by Zhao and Last (1996). A 70 mg aliquot of seedlings was harvested and frozen in liquid nitrogen. The frozen tissue was incubated at 80°C with 700 μl of 80% (v/v) methanol for 15 min before centrifugation at 13,000 g for 5 min. A 500 μl aliquot of supernatant was transferred to a new tube and evaporated to dryness at room temperature. The residue was resuspended in 100 μl of distilled water and extracted twice with 100 μl of chloroform. The pooled organic phase was evaporated, and the residue was resuspended in 15 μl of chloroform and spotted onto a silica TLC plate (20 cm×20 cm×250 μm, 60 Å porosity, 10–12 μm particle size, Whatman). The TLC plate was developed with chloroform:methanol (9:1), and visualized on a UV transilluminator.

Statistical analysis
Analysis of variance and a post hoc least significant difference test (SPSS 15.0, SPSS Inc., Chicago, IL, USA) were used to determine statistical significance between plant strains and treatments. All differences quoted in the text were significant at 0.05 or better.

Results
Antioxidant status of the vtc mutants
To characterize the effect of ascorbate deficiency on the small molecule antioxidants, 2-week-old vtc and cad2 seedlings were used. Under the growth conditions used in these experiments, there was no difference in plant size between wild-type and mutant plants. The total and reduced ascorbate concentrations (Fig. 1A) in all of the vtc mutants were significantly lower than in the wild type, with vtc2-1, vtc3, and vtc2-3 having the lowest at ~28–35% of the wild type. The other vtc mutants had higher total ascorbate concentrations ranging between 55 and 78% of the wild-type level. The ascorbate oxidation state did not vary between vtc mutants. This pattern is similar to that reported for soil-grown plants (Conklin et al., 2000). The GSH-deficient cad2 mutant and the wild-type and vtc2-1 seedlings treated with BSO had similar ascorbate contents to the corresponding untreated plants. Therefore, under these growth conditions, GSH deficiency did not affect ascorbate pool size or oxidation state.

The total GSH content (Fig. 1B) of the vtc mutants was similar to that of the wild type, with the exception of vtc2-3 and vtc4, which were lower and higher than the wild type, respectively. As expected, cad2 (Cobbett and May, 1997) and the wild-type and vtc2-1 seedlings treated with BSO had lower total GSH concentrations, ranging from 25 to 40% of the wild-type total GSH content. Approximately 75% of the total GSH in the wild type was present in the reduced form and it was significantly more reduced (>90% of total) in all of the other seedlings, with the exception of vtc2-1, vtc2-3, and vtc4. Therefore, the GSH pool was only affected to a small degree in the vtc mutants. Similarly, the α-tocopherol content of the vtc and cad2 seedlings was not different from that of the wild type (Fig. 1C).

The activities of enzymes involved in the ascorbate–GSH cycle, MDHAR, DHAR, and GR, were measured along with the hydrogen peroxide-metabolizing enzymes APX, peroxidase, and catalase (Table 1). MDHAR activity was significantly higher in vtc2-1, vtc2-2, vtc2-3, and vtc4 than in the wild type. DHAR activity was significantly higher in vtc3 and cad2. GR activity was lower in vtc2-3 and cad2 compared with the wild type. APX activity in vtc1 was more than doubled and the APX activity of vtc2-1 was also significantly higher than in the wild type. In contrast, vtc3 showed much lower APX activity, at ~30% of wild-type activity. vtc1 and vtc2-1 also showed much higher peroxidase activity than the wild type, although in APX the highest
Peroxidase activity was observed in vtc2-1, which had >2-fold higher activity than the wild type. Increased peroxidase activity was also measured in vtc4. Catalase activity did not differ between the wild type and all of the other mutants.

Peroxidase activity in vtc1, vtc2-1, and vtc4

Peroxidase was investigated further by fractionating leaf homogenates into soluble and ionically bound (cell wall) fractions (Fig. 2A). The soluble peroxidase activity was significantly higher in vtc2-1 seedlings and vtc2-1 seedlings treated with BSO. The elevated activity observed in vtc1 was just outside the statistically significant range. Greater differences in activity were observed for the ionically bound peroxidases, and vtc1, vtc2-1, vtc4, and BSO-treated wild-type and vtc2-1 seedlings showed significantly higher activity. The activity was highest in the BSO-treated vtc2-1 seedlings, which showed almost 3-fold higher activity than the wild type. The cad2 mutant did not have increased ionically bound peroxidase activity. Across all strains there was a significant negative correlation ($r = -0.61, P < 0.05$) between ascorbate concentration and ionic peroxidase activity (Fig. 2B).

Seedlings were treated with ascorbate and l-galactono-1,4-lactone (L-GalL) for 24 h to determine if the increased ionically bound peroxidase activity observed in untreated vtc1 could be reversed by ascorbate feeding. Contrary to expectation, ascorbate feeding caused a large increase in ionically bound peroxidase activity in both the wild type and vtc1 (Fig. 2C). Feeding L-GalL, which is the immediate biosynthetic precursor of ascorbate (Smirnoff et al., 2001), had no effect. Ascorbate feeding also induced ionically bound peroxidase activity in all the other vtc mutants (data not shown). Ascorbate feeding increased leaf ascorbate to four times the wild-type level, while L-GalL increased ascorbate to wild-type levels in vtc1 and doubled ascorbate concentration in wild-type leaves (data not shown). Conversely, L-GalL is converted to ascorbate intracellularly via the normal biosynthetic pathway, and the smaller increase over 24 h was not sufficient to alter apoplastic peroxidase activity. To investigate whether the variation in peroxidase activity was related to the cellular redox status of the seedlings, an oxidative stress treatment consisting of 50 mM hydrogen peroxide was applied to 2-week-old seedlings for 24 h. The extractable peroxidase activity was then measured. These treatments had no significant effect on ionically bound peroxidase activity. A cellulose biosynthesis inhibitor (2,6-dichlorobenzonitrile, DCB) was used to investigate whether peroxidase activity was influenced by alteration of the cell wall structure (Fig. 2D). Ionically bound peroxidases were significantly increased by between 2- and 4-fold in the wild type and vtc1. Soluble peroxidase also increased, but not significantly.
Transcript levels of endochitinase, GST6, PR1, and PR2 in the vtc mutants and cad2

Quantitative PCR (qPCR) was used to compare the transcript levels of endochitinase, PR1, PR2, and GST6 between the wild type, vtc mutants, and cad2. Additionally, the effect of GSH deficiency and combined ascorbate and GSH deficiency on transcript levels was assessed by using the cad2 mutant and BSO-treated plants (Fig. 3A–C). Comparison of endochitinase transcript levels in untreated (control) seedlings showed that vtc1, vtc2-1, vtc2-2, and vtc2-1+BSO, but not cad2, had significantly higher levels compared with the wild type (Fig. 3A). No endochitinase transcript was detected in the wild-type BSO-treated mutants but the increase was smaller in cad2. Ascorbate increased endochitinase transcripts compared with the control in all strains. Similarly to endochitinase, PR1 transcript levels were higher in the vtc mutants. BSO-treated wild-type seedlings showed a significantly decreased transcript level compared with the untreated wild type (Fig. 3C). SA significantly increased the PR1 transcript level in all strains tested, while ascorbate, paraquat, and UV-C did not increase PR1 transcripts relative to control in the wild type, vtc1, and vtc2-1. However, paraquat increased PR1 transcripts in vtc2-2 and cad2, and ascorbate increased PR1 transcripts in cad2. The PR2 transcript level was significantly higher in vtc1 and vtc2-1 but not in vtc2-2 or cad2 (Fig. 3C). Ascorbate increased the PR2 transcript level in vtc2-1 and cad2, while paraquat increased PR2 in the wild type, vtc2-1, vtc2-2, and cad2. SA only increased PR2 in vtc2-1 and vtc2-1+BSO. UV-C significantly decreased PR2 transcript levels in all strains compared with control. In contrast to the three PR genes, GST6 transcript levels in vtc1 and vtc2-2 did not differ from those of the wild type (Fig. 3D).

Interestingly, vtc2-1 transcript levels were very low in vtc2-1 and vtc2-1+BSO compared with the wild type, suggesting that this gene is specifically affected in the vtc2-1 allele. With the exception of vtc2-1, GST6 transcript levels were significantly increased by paraquat but not by SA or UV-C in the vtc and cad2 mutants.

Accumulation of camalexin in the vtc mutants

Accumulation of the phytoalexin camalexin is induced by pathogens and oxidative stress (Zhao et al., 1998). Camalexin in wild-type plants and mutants was therefore investigated by TLC. Camalexin was identified by its blue fluorescence and induction by silver nitrate (Zhao et al., 1998). Silver nitrate induced camalexin accumulation in all wild type, vtc mutants, and cad2. Without silver nitrate induction, a camalexin spot was visible for vtc1 and the three vtc2 mutants but not for vtc3, vtc4, and cad2 (Fig. 4).

Discussion

The effect of ascorbate deficiency on antioxidant status

Ascorbate concentration was decreased in vtc1 and all three vtc2 alleles as previously reported (Conklin et al., 1996, 2000). Under the growth conditions used in this experiment, the ascorbate content of the GSH-deficient cad2 mutant, and of plants treated with BSO to induce GSH deficiency, was not affected. Conversely, the GSH pool and reduction state were not greatly affected in the vtc mutants. In contrast, mature vtc1 and vtc2-1 plants have 20–30% more GSH than the wild type, which is more reduced (Veljovic-Jovanovic et al., 2001; Muller-Moule et al., 2004; Pavet et al., 2005). α-Tocopherol, the major tocopherol isomer in A. thaliana leaves, was unaffected in the vtc and cad2 mutant seedlings, although Kanwischer et al. (2005) found that soil-grown vtc1 and cad2 mutant plants had ~50% higher α-tocopherol content than the wild
type. Kanwischer et al. (2005) also found that an α-tocopherol-deficient mutant, vte1, had elevated ascorbate and GSH levels, whereas transgenic plants overexpressing VTE1, a gene encoding a tocopherol biosynthesis enzyme (tocopherol cyclase), showed a reduction in ascorbate levels compared with the wild type. Therefore, in 2-week-old seedlings grown under the conditions used in this investigation, GSH or α-tocopherol do not increase in the vtc mutants to compensate for the low ascorbate levels, and likewise there was no increase in ascorbate in the cad2 mutant and BSO-treated seedlings to compensate for the GSH deficiency. However, in plants grown under higher light intensity, compensation does occur. Similarly, the activities of the ascorbate–GSH cycle enzymes MDHAR, DHAR, and GR were not greatly affected in any of the mutants. In contrast, APX activities were elevated in vtc1 and vtc2-1. Increased APX has been reported previously in vtc1 (Veljovic-Jovanovic et al., 2001). This is unlikely to be due solely to the low ascorbate levels since both vtc2-3 and vtc3 had lower ascorbate content than vtc1 yet showed no increase in APX activity. Overall, the results from this study and others show that under relatively non-stressful conditions, ascorbate deficiency is not compensated by changes in antioxidants other than APX, but under higher light intensities GSH pool size does increase in low ascorbate mutants. This conclusion is also supported by the lack of increase in the transcripts of genes encoding antioxidants in vtc1 compared with wild-type plants.
It therefore seems that the vtc mutants are not under severe oxidative stress under the growth conditions used in these experiments. This is confirmed by lack of induction of GST6/At2g47730. In contrast, the GST6 transcript level was consistently lower than that of the wild type in vtc2-1, which has the lowest ascorbate concentration. This gene is considered to be responsive to oxidative stress (Kovtun et al., 2000; Desikan et al., 2001), and this was confirmed by paraquat induction. It should be noted that the simple measurements of antioxidant pool sizes and enzyme activities do not rule out the possibility that turnover of these antioxidants could be altered in the mutants.

**Increased peroxidase activity in vtc mutants**

Higher ionically bound peroxidase activity in vtc1, vtc2-1, and vtc4 seedlings suggests that these lines have higher cell wall peroxidase activity, since this is the main...
location of this fraction (Fry, 1988). Higher soluble peroxidase activity has been noted in vtc1 (Veljovic-Jovanovic et al., 2001) and also in low ascorbate A. thaliana plants expressing antisense L-galactose dehydrogenase, an enzyme involved in ascorbate biosynthesis (Gatzek et al., 2002). Additionally, soil-grown vtc1, vtc2-1, and vtc2-2 plants have increased cell wall peroxidase (NS, unpublished results). Together, these results from diverse mutants suggest that increased cell wall peroxidase is caused by low ascorbate. Cell wall peroxidase has been implicated in determining cell wall structure and mechanical properties through peroxidative cross-linking of wall proteins and polysaccharides (Brady and Fry, 1997), and in cross-linking monolignols during lignification (Blee et al., 2003). These processes also contribute to localized wall strengthening in response to pathogens (Lamb and Dixon, 1997). It has also been suggested that wall peroxidase contributes to the oxidative burst, since under certain conditions peroxidase can generate hydrogen peroxide. Antisense plants with reduced wall peroxidase have decreased apoplastic hydogen peroxide and are more sensitive to a range of virulent and avirulent pathogens (Bindschedler et al., 2006). Given the increased resistance of vtc1 and vtc2-1 to virulent pathogens (Barth et al., 2004; Pavet et al., 2005), along with increased PR protein gene transcripts and camalexin production, it is interesting that ascorbate deficiency also induces cell wall peroxidase. Transcriptome analysis of vtc1 showed that the transcript level of one peroxidase (At2g37130) was slightly higher in vtc1, while another peroxidase showed a slightly lower transcript level (Pastori et al., 2003). To explore the role of ascorbate further, plants were supplemented with ascorbate or with a biosynthetic precursor, L-GalL (Pallanca and Smirnoff, 1999). Surprisingly, these treatments, while increasing ascorbate pool size, did not decrease peroxidase; instead, ascorbate feeding caused a large increase. In agreement with this effect, transcriptome analysis of vtc1 plants supplemented with ascorbate revealed that another peroxidase (At1g564100) had the highest fold increase of all genes (Pastori et al., 2003). Peroxidase induction by both ascorbate feeding and ascorbate deficiency requires explanation. It has been suggested that the pool size and redox state of apoplastic ascorbate (controlled by ascorbate oxidase) has a role in signal transduction (Pignocchi and Foyer, 2003; Pignocchi et al., 2006). Since exogenous ascorbate might become oxidized, the apoplast will have higher than normal dehydroascorbate concentrations which may then result in perception of an oxidized apoplast. Ascorbate deficiency and the resulting low apoplastic ascorbate (Veljovic-Jovanovic et al., 2001) could also signal an oxidized apoplast, leading to the same response. L-GalL feeding did not act in the same way since ascorbate is generated intracellularly, and reversal of peroxidase induction was not seen because 24 h is probably not sufficient time for significant turnover of wall peroxidases. Since at least some of the mutants are affected in synthesis of mannosyl and L-galactosyl residues in the cell wall (Conklin et al., 1999, 2006) and are also affected in cellulose synthesis (Lukowitz et al., 2001), an alternative hypothesis to explain peroxidase induction is that defence responses are induced by disruption of cell wall synthesis or structure. The mutant cev1 is affected in cellulose synthase and shows increased pathogen resistance mediated by the jasmonate and ethylene pathways (Ellis and Turner, 2001; Ellis et al., 2002). This effect was mimicked by the cellulose synthase inhibitor 2,6-dichlorobenzonitrile (Ellis et al., 2002). Therefore, the effect of 2,6-dichlorobenzonitrile on peroxidase was tested. It also induced peroxidase in the wild type and vtc1. Therefore, although part of the increased peroxidase activity could be caused by perturbed cell wall composition in some of the mutants, the overall negative correlation between peroxidase and ascorbate, along with the same effect in a range of low ascorbate plants expressing antisense L-galactose dehydrogenase (Gatzek et al., 2002), suggests ascorbate deficiency itself to be the most likely explanation.

**The relationship between ascorbate and pathogen responses**

A range of pathogen response genes (endochitinase/PR3/At2g43570, PR1/At2g14610, and PR2/At3g57260), previously shown to have higher transcript level in vtc1 (Pastori et al., 2003), were confirmed by qPCR to have higher transcript levels in vtc1 as well as in vtc2-1 and vtc2-2. The similar pattern in three different vtc mutants confirms that the increased transcript level of PR genes is most probably caused by ascorbate deficiency. However, as discussed above, vtc1 shows the biggest response and is also affected in cell wall composition, which may trigger some aspects of the pathogen response system. The same endochitinase and PR1 transcripts were also higher in a cytosolic APX knockout mutant (Davletova et al., 2005), providing further evidence for a link with ascorbate. Endochitinase was also highly responsive to UV-C, SA, and paraquat, as well as ascorbate. The increase in endochitinase gene transcript level after ascorbate
treatment may be related to DHA generation in the cell wall, as discussed above for peroxidase activity. GSH is linked to pathogen responses, particularly via effects on the redox state of cysteine in NPR1, a protein involved in PR gene transcript level (Despres et al., 2003). A decrease in GSH or an increase in its oxidation state would be predicted to increase the oxidation state of NPR1, thus decreasing the PR transcript level. Accordingly, in cad2 and BSO-treated wild-type plants, which had similar reductions in GSH concentration caused by lower γ-glutamylcysteine synthetase activity, endochitinase, PR1, and PR2 transcript levels were similar to the wild type or slightly decreased. In agreement, all three of these genes had lower transcript levels in cad2 and rax1, which is another γ-glutamylcysteine synthetase mutant (Ball et al., 2004). Although BSO decreased the transcript levels of all three PR genes, endochitinase and PR2 transcript levels were still high in vtc2-1 treated with BSO, suggesting that GSH deficiency cannot prevent an increased PR gene transcript level in an ascorbate-deficient plant. Camalexin accumulation in vtc1 and the vtc2 alleles further confirms that pathogen responses are activated in these lines. As with PR gene transcript level, GSH deficiency in cad2 did not induce camalexin and it remained responsive to camalexin induction by silver nitrate. Both PR gene transcript levels and camalexin accumulation are SA dependent (Zhao and Last, 1996; Pieterse and Van Loon, 2004) so the increased SA concentration in vtc1 (Barth et al., 2004) could explain their induction. The mechanism of induction of camalexin by silver nitrate has received little comment. Although it is used as an inhibitor of ethylene action (Davies et al., 1988), it rapidly oxidizes ascorbate. The silver nitrate treatment caused a large reduction in ascorbate (data not shown), so its ability to elicit camalexin synthesis could also be related to ascorbate oxidation in the apoplast. The GST6 transcript level was unaffected in the vtc mutants compared with the wild type. It responded to paraquat but not to SA treatment. GST6 is known to respond to hydrogen peroxide (Kovtun et al., 2000; Desikan et al., 2001), showing that ascorbate deficiency does not result in oxidative stress under the growth conditions used but does lead to altered transcript levels of PR genes.

**Conclusions**

Four ascorbate-deficient vtc mutants were compared under ‘non-stressful’ conditions. This showed that they do not suffer from severe oxidative stress, and that other components of the antioxidant system show little compensatory change under the relatively non-stressful growth conditions used. In contrast, the vtc mutants, particularly vtc1 and vtc2, had increased transcript levels of PR genes, increased cell wall peroxidase activity, and accumulated camalexin. Considering the previously reported SA accumulation and increased resistance to virulent *Pseudomonas syringae* and *P. syringae* (Barth et al., 2004; Pavet et al., 2005), it is clear that ascorbate influences some of the signalling systems involved in pathogen resistance. The redox states of ascorbate and GSH are at least partly linked through the ascorbate–GSH cycle (Noctor and Foyer, 1998), and Ball et al. (2004) have shown that, in contrast to the vtc mutants, GSH-deficient mutants have lower transcript levels of PR proteins and are more susceptible to virulent *P. syringae*, and suggested that ‘redox tone’ influences pathogen responses. It is clear that there are complex links between the two major small molecule antioxidants in plants and that overall ‘redox tone’ is influenced by both these, leading to modulation of signalling processes. It should also be noted that alterations in cell wall composition, particularly in vtc1, could contribute to induction of pathogen responses.

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**References**


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