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Effects of Leaf Ascorbate Content on Defense and Photosynthesis Gene Expression in *Arabidopsis thaliana*

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ABSTRACT

Ascorbate deficiency in the *Arabidopsis thaliana vtc1* mutant had no effect on photosynthesis, but modified defense pathways. The ascorbate content of *vtc1* leaves was increased 14-fold after 10 m*M* ascorbate was supplied, without a concomitant change in redox state. High ascorbate modified the abundance of 495 transcripts. Transcripts encoding dehydroascorbate reductase, pathogenesis-related protein 1, and a peroxiredoxin were decreased, whereas those encoding salicylate induction-deficient protein 1, Cu,Zn superoxide dismutase, iron superoxide dismutase, metallothionein, and glutathione transferases were increased. Catalase transcripts were unaffected, but ascorbate peroxidase isoforms *APX1* and *tAPX* were slightly decreased and *sAPX* transcripts increased. A number of nuclear encoded transcripts for photosynthetic electron transport components were repressed as a result of ascorbate accumulation, whereas those that were chloroplast-encoded were increased. High ascorbate caused decreases in mRNAs encoding chloroplast enzymes such as fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase that are activated by reduced thioredoxin. In contrast, others, such as glucose 6-phosphate dehydrogenase, whose activity is inactivated by reduced thioredoxin, were repressed. Together, these results show that ascorbate is involved in metabolic cross-talk between redox-regulated pathways. The abundance of this antioxidant provides information on redox buffering capacity that coordinates redox processes associated with the regulation of photosynthesis and plant defense. *Antioxid. Redox Signal.* 5, 23–32.

INTRODUCTION

L-ASCORBIC ACID is the most abundant low-molecular-weight hydrophilic antioxidant in plants, where it has many functions (19, 24). For example, it is an essential cofactor for a large number of plant enzymes, such as the 2-oxoacid-dependent dioxygenases (1). It also has several key roles in photosynthetic energy partitioning (1). Ascorbate is made in mitochondria and accumulates to very high concentrations in leaves, fruits, and actively growing tissues (19, 24). It is one of the most important cellular buffers protecting against the high oxidative load that accompanies rapid metabolism. A major function of ascorbate is the destruction of active oxygen species, particularly hydrogen peroxide (H_2O_2). H_2O_2 is produced at very high flux rates by a number of metabolic processes in plants, particularly photosynthesis and photorespiration (6, 20).

In contrast to ascorbate peroxidases (APX) that use ascorbate to reduce H_2O_2 to water, catalases (CAT) convert H_2O_2 to oxygen and water without consuming reducing power (8, 11, 13, 27–29, 31). Three CAT genes (*Cat1*, *Cat2*, and *Cat3*) are found in the *Arabidopsis* genome. Developmental and environmental triggers regulate the expression of these genes (8, 27, 28). The pattern of *Cat2* expression is consistent with a role in the peroxisomal degradation of photorespiratory H_2O_2. A small gene family also encodes APX. Cytosolic,

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chloroplast stromal and thylakoid forms, peroxisomal and mitochondrial isoforms have been identified and characterized (11, 13, 29, 31).

There is an extensive literature on APX and the antioxidant function of ascorbate, but many other important functions of ascorbate are less well characterized. Ascorbate has long been implicated in the control of mitosis and cell growth (19). It has recently been demonstrated that, like H_2O_2, ascorbate modifies gene transcription and mediates plant responses to the environment (4, 18). Changes in gene expression result from modified amounts of ascorbate rather than from a general change in cellular redox balance (21, 25). Ascorbate deficiency triggers changes in plant development via hormonal signaling pathways (21). It also induces major changes in plant defense networks activating genes normally associated with pathogenesis resistance rather than antioxidative enzymes (21).

The *Arabidopsis thaliana* vitamin C (*vtc*) *1* mutant, which has 70% lower leaf ascorbate contents than the wild type, has proven to be a very useful tool in the elucidation of ascorbate signaling in plants (21). This mutant has impaired ascorbate biosynthesis capacity (2, 3). The *vtc1* mutant is smaller than the wild type and shows delayed flowering and accelerated senescence (25), but it does not accumulate H_2O_2, and the redox states of leaf ascorbate, glutathione, and tocopherol pools are unchanged compared with the wild type (21, 25). In a previous study, we have analyzed the link between ascorbate deficiency and the *vtc1* phenotype through an integrated approach involving transcriptome analysis, physiological measurements, and biochemical assays (21). Transcriptome data were obtained from five replicates of columbia (Col) 0 leaves and five replicates of *vtc1* leaves using the Affymetrix Gene Chip technology and Expression Analysis Software (GENECHIP ver. 3.3). We then validated and extended the primary analysis by reassembling and reanalyzing the data to treat all replicate analyses as a single experiment using dChip. A comparison of the transcriptome of rosette leaves from *vtc1* with those of wild type (Col 0) showed that 171 transcripts were significantly modified (15, 21). Of these, 138 represented either genes of known function or genes to which a function could putatively be assigned on the basis of homology. These included 54 encoding DNA-binding proteins (21 genes), or proteins implicated in cell-cycle control (12 genes), signaling (9 genes), and developmental (12 genes) processes. Transcript levels for 42 proteins involved in metabolism were modified, including enzymes involved in carbon metabolism (15 genes), cell-wall metabolism (10 genes), lipid metabolism (7 genes), anthocyanin synthesis (4 genes), indole metabolism (4 genes), and sulfur assimilation (2 genes).

There were no changes in transcripts of genes encoding components of the photosynthetic electron transport system in *vtc1* leaves. The abundance of *rbcL* transcripts encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) were, however, decreased by ~20% in the *vtc1* mutant (21). Similar decreases were observed in the amounts of Rubisco protein and Rubisco maximal activities, but the activation state of the enzyme was higher in *vtc1* than in the Col 0 controls (21). As a result, the measured rates of photosynthetic CO_2 fixation were similar in Col 0 and *vtc1* (25).

In our previous studies (21, 25), we have shown that ascorbate deficiency in *vtc1* causes slow growth and late flowering. This phenotype was not caused by changes in photosynthesis or increased oxidative stress (25). Using array technologies, we were able to obtain a molecular signature for ascorbate deficiency in plants (21). Ascorbate deficiency led to extensive "cross-talk" between different pathways involved in defense against biotic and abiotic stresses. In particular, pathogenesis-related (PR) proteins and other lytic enzymes were greatly increased in *vtc1* (21). We were able to show that growth and development were constrained in *vtc1* because the balance between abscisic acid and gibberellic acid (GA) signaling was modified (21). Abscisic acid was significantly higher in *vtc1* leaves than the wild type. In the present study, we have explored the effect of restoring the wild-type ascorbate content to *vtc1* leaves. In these experiments, we have specifically modified leaf ascorbate contents, and not the redox state of the ascorbate pool, to explore the hypothesis that the extent of accumulation of leaf ascorbate is involved in metabolite cross-talk between pathways comprising redox reactions. We have analyzed the effect of short-term enhancement of the leaf ascorbate pool on the leaf transcriptome with particular reference to genes involved in defense and in photosynthesis. The data presented here confirm that ascorbate is an important signaling molecule in plants and that the amount of ascorbate in leaves modulates the expression of specific sets of defense and photosynthesis genes.

MATERIALS AND METHODS

Plant material

The following experiments were carried out using the *Arabidopsis thaliana* wild-type Col 0 and the *vtc1* mutant (2). Plants were grown for 7–10 weeks in pots containing a mixture of compost/sand (3:1) in controlled environment chambers [8-h photoperiod, 200 μmol of quanta m^{-2} s^{-1}, 60% (vol/vol) relative humidity, and day/night temperature of 23°C/18°C].

Ascorbate feeding

Leaf discs (1 cm in diameter) were excised from rosette leaves under water. Replicates of eight leaf discs per treatment were incubated in 10 m*M* MOPS buffer, pH 6.0, either alone or containing 5 m*M* or 10 m*M* ascorbate for 16 h in the dark. Following incubation, leaf discs were then rinsed, dried, and used in the following experiments.

Analysis of ascorbate and dehydroascorbate

Ascorbate and dehydroascorbate were extracted and assayed using ascorbate oxidase, as described (5).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Fully developed leaves were collected at random from rosettes and pooled for RNA extraction and mRNA purification by prescribed methods (http://afgc.stanford.edu/afgc-

array-rna.html). Genomic DNA was removed from the RNA samples by adding 1 µl of 10 × DNase I Reaction Buffer, 1 µl of DNAse I (Invitrogen), Amp Grade (1 U/µl), and diethylpyrocarbonate-treated water to 1 µg of RNA sample to a final volume of 10 µl. After 15 min of incubation, DNAse I was inactivated by the addition of the 25 m*M* EDTA, followed by incubation at 65ºC for 10 min. Synthesis of the first strand of cDNA was performed by adding 1 µl of oligo$(dT)_{12-18}$ and 0.4 µl of 25 m*M* dNTPs (equimolar solution of dATP, dCTP, dGTP, and dTTP at pH neutral) to 1 µg of total RNA. The mixture was then heated to 65ºC for 5 min before being quickly chilled on ice. A brief centrifugation was followed by the addition of 4 µl of 5 × First-Strand buffer (250 m*M* Tris-HCl buffer, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$) and 2 µl of 0.1 *M* dithiothreitol, and the equilibration of the samples at 42ºC for 2 min. The transcription initiated from oligo dT primers was then carried out with 200 U of the Reverse Transcriptase SuperScript II (GIBCO) for 50 min at 42ºC. The reaction was finally inactivated by heating the mixture at 70ºC for 15 min. Samples were stored at −20ºC until the PCR reaction. PCR reactions were performed with specific primers for each of the genes analyzed (Table 1). *Arabidopsis* Actin 2/7 was used as an internal control in order to normalize each sample for variations in the amount of initial RNA. The PCR mix included 1 µl of the template, 5 µl of 10 × PCR buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl), 0.4 µl of 25 m*M* dNTPs, 1 µl of each solution of 10 m*M* of primers (forward and reverse), 1.5 µl of 50 m*M* $MgCl_2$, and 2 U of *Taq* polymerase. Sterile distilled water was added to a final volume of 50 µl. PCR was carried out in a programmable Robocycler at each specific annealing temperature for all genes (Table 1). The products of PCR amplification resulted in a single band of the predicted molecular weight. These were analyzed on 1.5% agarose gel against a 1-kb DNA ladder (MBI Fermentas).

Quantitation of PCR products

The quantitation of PCR products was performed using Actin 2/7 as an internal control. For an accurate comparison of the transcript levels in control and treated samples, PCR reactions were terminated when the products from both Actin 2/7 and the gene of interest were detectable and were being amplified within the exponential phase. The exponential phase of amplification occurred at a specific number of PCR cycles when the reaction components were still in excess and the PCR products were accumulating at a constant rate (Table 1). RNA extractions and RT-PCR experiments were carried out at least three times.

Microarray analysis

Two leaf RNA samples (eight leaf discs per sample) from *vtc1* control and two from *vtc1* incubated with 10 m*M* ascorbate were prepared as described above (http://afgc.stanford.edu/afgc-array-rna.html). Two cDNA plates provided by Stanford University were hybridized with both *vtc1* and *vtc1* plus ascorbate RNA samples (http://afgc.stanford.edu/afgc_html/site2.htm). On plate 1, *vtc1* was stained with a fluorofor (Cy5) that fluoresces at a red wavelength (λ = 635 nm), and *vtc1* plus ascorbate was stained with a second fluorofor (Cy3) that fluoresces at a green wavelength (λ = 532 nm). The two stained samples were hybridized on the same *Arabidopsis* cDNA plate. For a detailed description of the microarray experiment, please see http://afgc.stanford.edu/afgc_html/AFGCProtocols-Aug2001.pdf. On plate 2, the dye tag swapped such that *vtc1* responds to red wavelengths and *vtc1* plus ascorbate responds to green wavelengths. These two hybridizations thus formed a "dye-swap" plate pair. The hybridized plates were scanned, and spot intensities in each wavelength were generated by an Axon GenePix system for each plate of the pair. Following visual inspection of the array images and the associated data, the experimental data were normalized using the mechanisms described by Yang *et al.* (30; available at www.stat.Berkeley.EDU/users/terry/zarray/Html/image.html and http://citeseer.nj.nec.com/article/yang00comparison.html). The data for each slide were plotted as an *M* versus *A* plot, where $M = \log_2(R/G)$, $A = \log_2(\sqrt{(RG)})$, and *R* and *G* are the red and green intensities of each spot, respectively. This process involves the normalization of the data, taking into account print tip variations (often referred to as Block variations) using a robust Lowess smoothing to remove variation due to print tip wear and size, followed by a robust scaling of each print tip. The robust scaling suggested by Yang *et al.* (30) was a_i^2 where $a_i = MAD_i / \sqrt[I]{[\prod_{i=1}^{I} MAD_i]}$, $MAD_i = \text{median}_j \{|M_{ij} - \text{median}_j(M_{ij})|\}$, and MAD is the mean absolute deviation. In this manner, each plate is normalized, and following this normalization process, a normalized *M* versus *A* plot is produced in which the differentially expressed proteins appear as outliers of the normalized *M* versus *A* plot. The normalization process was undertaken on each of the dye-swap plates and the values of *R*/*G* inverted on the second plate to give a direct comparison. The mean expression level (*i.e.*, mean *M*), for each spot, was calculated from the normalized plates. An arbitrary cut-off of $|\text{mean } M| \geq 0.95$ was chosen as an initial boundary level above which reasonable confidence could be given.

RESULTS

Ascorbate uptake

Arabidopsis leaf discs rapidly took up ascorbate over the 16-h incubation period (Table 2). The ascorbate present in the bathing solutions was not oxidized during the experiment (data not shown). *Vtc1* leaf discs took up greater amounts of ascorbate than the Col 0 controls, but Col 0 discs still contained more ascorbate than those of the mutant by the end of the experiment (Table 2). Overall, the discs incubated with 5 m*M* ascorbate, took up ~7 µmol/g fresh weight, whereas those incubated with 10 m*M* ascorbate took up 13–15 µmol/g fresh weight. The capacity for ascorbate uptake was similar in the control and mutant leaf discs. The calculated rate of ascorbate uptake was higher when discs were supplied with 10 m*M* ascorbate (0.9 µmol/g fresh weight/h) than with 5 m*M* ascorbate (0.4 µmol/g fresh weight/h). The redox state of ascorbate pool was high in discs incubated either in buffer alone or in buffer with ascorbate, being ~90–92% reduced in all cases (Table 2). After incubation with 10 m*M* ascorbate, Col 0 discs had five times more ascorbate than discs incubated in buffer alone (Table 2). In comparison, *vtc1* discs had ~14 times more ascorbate. In all the following experiments

TABLE 1. PRIMER SEQUENCES OF *ARABIDOPSIS* CATALASE (*CAT*), ASCORBATE PEROXIDASE (*APX*), AND ACTIN-1 (*ACT*) GENES USED IN RT-PCR ANALYSIS

Name of sequence	*Accession number*	*Name of primer*	*Sequence (5′-3′)*	*Annealing temperature (°C)*	*Number of cycles*
Catalase 1 (*Cat1*)	U43340	A-CAT1-F	ATGCCTGTCGGATGAGGA	50	25
		A-CAT1-R	GACAATAGGAGTTGTAGGGTA		
Catalase 2 (*Cat2*)	X94447	A-CAT2-F	TTTGGAAGAAGATGCAATTC	50	25
		A-CAT2-R	GCAGGTGGAGTTGGATAC		
Catalase 3 (*Cat3*)	U43147	A-CAT3-F	ACTGATGAAGAGGCCAAGGT	50	25
		A-CAT3-R	GAGTTTGTAGGGGTGGGAAC		
Cytosolic ascorbate, peroxidase (APX1)	X59600	C-APX1-F	AAGGCTGTTGAGAAGTGC	46	35
		C-APX1-R	TTAAGCATCAGCAAACCC		
Cytosolic ascorbate, peroxidase (APX2)	X98275	C-APX2-F	TATTGCCGAGAAGCACTG	46	35
		C-APX2-R	ACTCCTTGTCAGCAAACC		
Thylakoid-bound, ascorbate peroxidase (tAPX)	X98926	t-APX-F	AATAGTTGCCTTGTCTGG	46	45
		t-APX-R	GGAATATATGATCACCACG		
Stromal ascorbate, peroxidase (sAPX)	X98925	s-APX-F	TGTTCCAGTTAGCTAGTG	46	45
		s-APX-R	GGTTGAGTAAATTAGGTGC		
Actin-1	M20016	ACT-F	GAGAAGATGACTCAGATC	46/50	25/35
		ACT-R	ATCCTTCCTGATATCGAC		

TABLE 2. ASCORBATE CONTENT AND REDOX STATE IN *Arabidopsis* COL 0 AND *vtc1* MUTANT LEAF DISCS

Plant material	*Ascorbate content in solution (mM)*	*Total ascorbate content in leaves (μmol/g fresh weight)*	*% reduced*
Col 0	0	4.0[a]	90%[a]
	5	10.3[b]	96%[a]
	10	19.3[c]	92%[a]
vtc1	0	0.99[a]	92%[a]
	5	8.3[b]	95%[a]
	10	13.6[c]	91%[a]

Arabidopsis Col 0 and *vtc1* mutant leaf discs were incubated in 0, 5, and 10 m*M* ascorbate for 16 h in the dark and the ascorbate content and redox state determined. Values are means of two experiments. Different letters indicate significantly different values. The percentage of reduction was calculated as ascorbate × 100/(ascorbate + dehydroascorbate).

comparisons of gene expression were performed on *vtc1* leaf disc samples prepared after incubation in buffer alone or in buffer containing 10 m*M* ascorbate.

Transcriptome analysis

Four hundred ninety-five transcripts showed changed expression as a result of the increase in ascorbate in the *vtc1* leaf discs (Fig. 1). Of these 236 were increased by twofold or above, whereas 259 genes were decreased by twofold or more. Of the genes to which known functions had been ascribed, 60 were involved in cell rescue and defense and 12 belonged to cell metabolism (Fig. 1). Sixteen had functions in transport, 13 in development, 21 in DNA binding, 3 in indole metabolism, 7 in cell cycle, 29 in signaling, 29 in photosynthesis and carbon metabolism, 12 in secondary metabolism, 38 in protein synthesis, 13 in lipid metabolism, and 13 in nitrogen and sulfur metabolism. Changes in transcript abundance of selected genes were confirmed by RT-PCR (21). In

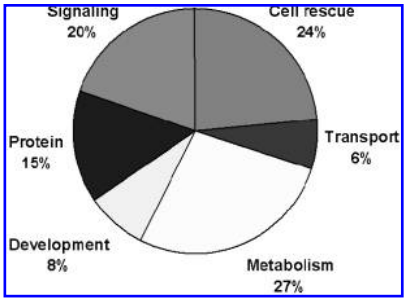

FIG. 1. Ascorbate-dependent modifications in the *Arabidopsis* transcriptome. Microarray analysis was used to compare leaf transcript abundance in the ascorbate-deficient *vtc1* leaves with those supplied with 10 m*M* ascorbate. *vtc1* plants were grown for 6 weeks in pots containing a mixture of compost/sand (3:1) in controlled environment chambers [8-h photoperiod, 200 μmol of quanta m^{-2} s^{-1}, 60% (vol/vol) relative humidity, and day/night temperature of 23°C/18°C]. Discs from fully developed leaves were incubated for 16 h in the dark in the absence or presence of 10 m*M* ascorbate. Discs were then collected at random, rinsed, dried, and pooled for RNA extraction and mRNA purification by prescribed methods (http://afgc.stanford.edu/afgc-array-rna.html). The chart shows transcripts that were significantly and reproducibly modified in abundance and assigned to functional categories.

the following analysis, we emphasize the effects of ascorbate on genes involved in defense and in photosynthesis.

The effect of ascorbate on antioxidant defense genes

Enhanced tissue ascorbate contents caused relatively few changes in the abundance of transcripts of known antioxidant defense genes (Fig. 2A; Table 3). Transcripts encoding dehydroascorbate reductase (At1g19570) and a peroxiredoxin (At3g06050) were decreased by ascorbate, whereas a Cu,Zn-superoxide dismutase (SOD; At5g18100) and an Fe-SOD (At4g25100) were increased. Ascorbate feeding induced

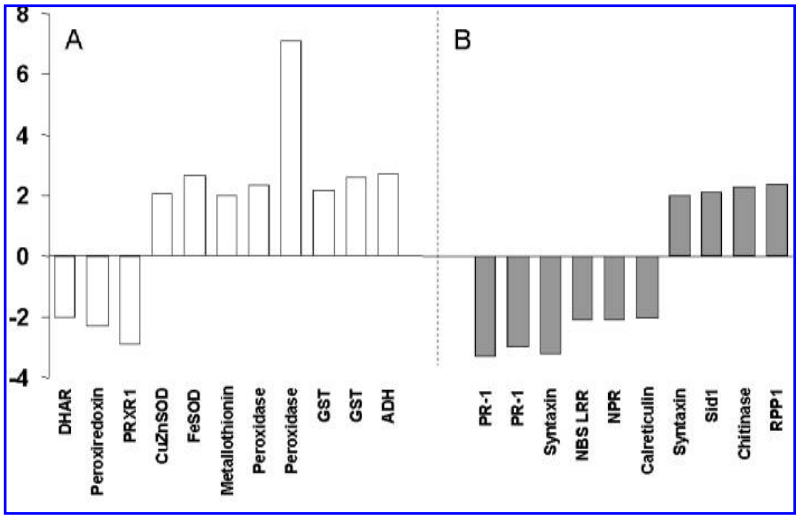

FIG. 2. High ascorbate-dependent modulation of the abundance of transcripts involved in antioxidant metabolism (A) and pathogen resistance (B). ADH, alcohol dehydrogenase; DHAR, dehydroascorbate reductase; GST, glutathione *S*-transferase; NBS LRR, central nucleotide-binding site, leucine-rich repeat; NPR, nematode-resistance protein; PR, pathogenesis-related; PRXR, peroxidase; RPP, resistance to *Pseudomonas parasitica*; Sid, salicylate induction-deficient.

transcripts encoding a metallothionein (At5g02380) and two glutathione transferases (At1g69930 and At1g78360). The situation was less clear with regard to peroxidase whose activity is known to be increased in *vtc1* compared with the wild type (25). The abundance of transcripts of one type of peroxidase (prxp1; At4g21960) was decreased by threefold by enhanced tissue ascorbate, whereas transcripts encoding other types of peroxidase (At4g26010 and At5g64100) increased by up to sevenfold (Fig. 2A).

As *CAT* and *APX* encode major antioxidants in photosynthetic cells that are essential for the efficient operation of photosynthesis, we decided to examine the effects of ascorbate on the transcripts encoding different isoforms of these enzymes in more detail (Fig. 3; Table 3). The array analysis revealed no effects of ascorbate content on the abundance of *Cat1* (At1g20630), *Cat2* (At4g35090), or *Cat3* (At1g20620) transcripts (Table 3). The absence of an effect of ascorbate on CAT transcript abundance was confirmed by RT-PCR (Fig. 3). Of the three isoforms, *Cat3* transcripts had the highest abundance at the end of the 16-h dark incubation period. Amounts of *Cat1* transcripts were lower than those of *Cat3*, whereas *Cat2* transcripts were almost undetectable (Fig. 3).

The array analysis revealed no significant (above or below twofold) effects of ascorbate content on *APX* transcript abundance. However, RT-PCR analysis (Fig. 3) revealed that increased tissue ascorbate caused subtle changes in the amounts of *APX1* (At1g07890), *tAPX* (thylakoid *APX;* At1g 77490), and *sAPX* (stromal *APX;* At4g08390) mRNA. The abundance of *tAPX* and *APX1* transcripts was high in Col 0 and *vtc1* discs in the absence of added ascorbate (Fig. 3). In contrast, *sAPX* transcripts were below the level of detection in this situation. In leaf discs incubated with ascorbate, the amount of *APX1* transcripts was decreased in *vtc1*, but not in Col 0 (Fig. 3). *sAPX* transcripts showed a slight increase in *vtc1* and Col 0 discs incubated with 10 m*M* ascorbate. The amount of *tAPX* transcripts was decreased in *vtc1* and Col 0 discs incubated with 10 m*M* ascorbate compared with discs incubated in buffer alone. *APX2* (At3g09640) transcripts could not be detected in any conditions in these experiments (data not shown).

The effect of ascorbate on genes encoding PR proteins and associated genes

Vtc1 leaves show constitutive induction of a number of PR transcripts, including *PR-1* (At2g14610), *PR-2* (At3g57260), and a thaumatin-like *PR-5* (At1g75040), as well as β-1,3-glucanase and an endochitinase (21). The *vtc1* mutant was isolated via its hypersensitivity to atmospheric ozone (2). Ozone treatment is known to induce PR proteins, and this is thought to occur through activation of signaling elements involved in the pathogen response, such as the accumulation of active oxygen species and the ensuing synthesis of salicylic acid (10, 14). This view is supported, for example, by coordinated expression of *PR-1* and phenylalanine ammonium lyase (*PAL*) in poplar exposed to ozone (12). The absence of *PAL* upregulation in *vtc1* shows that low ascorbate is associated with induction of PR proteins through signaling pathways that are independent of active oxygen species and salicylic acid (21). The extreme hypersensitivity of *vtc1* to ozone (2) may be due to a potentiating effect of low ascorbate on defense induction. After 16 h of incubation with ascorbate, *PR-1* transcripts were repressed in *vtc1* (Fig. 2B), whereas *PR-5* and *PR-2* transcripts were not significantly changed. *PR-1, PR-2,* and *PR-5* transcripts do not therefore appear to be coordinately expressed in the *vtc1* mutant. *Eds5* (enhanced disease susceptibility) transcripts that encode a transmembrane protein classified as a multidrug and toxin extrusion protein are induced in *vtc1* by ascorbate. *Eds5* (At4g39030) is allelic to *Sid1* (salicylate induction-deficient; Fig. 2B). The *Arabidopsis sid1* mutant does not accumulate salicylic acid or PR-1, but still shows induction of other PR proteins in response to infection (17). There is no evidence for coordinate regulation of *PR-1, PR-2*, and *PR-5* in the ascorbate-treated *vtc1* mutant.

Other effects appear to be more complex where multigene families are involved. For example, one type of syntaxin (At4g17730), a protein associated with vesicle formation and excretion, was decreased in response to ascorbate, whereas another form (At3g52400) was increased (Fig. 2B).

The effect of ascorbate on genes associated with photosynthesis

At high light, mutants with low ascorbate become sensitive to photoinhibition because of limitations on reactions associated with photosynthesis (16). We have previously shown that the low concentrations of ascorbate in *vtc1* leaves are sufficient to maintain thermal energy dissipation and optimal rates of the Mehler-peroxidase reaction (6, 21, 25). Consistent with this view, the *vtc1* and wild-type transcriptomes were remarkably similar with regard to transcripts encoding photosynthetic proteins (21). The situation changed markedly, however, when *vtc1* discs were subjected to a large and rapid increase in ascorbate (Fig. 4). Ascorbate increased the abundance of transcripts encoding chloroplast encoded components of the electron transport system [PsaA, CP-47, and the photosystem II (PSII) 44-kDa reaction center protein] and decreased those encoded in the nucleus (Fig. 4A).

The array analysis revealed significant decreases in key transcripts encoding components of the noncyclic pathway of electron flow. These are photosystem I (PSI) (*subunit II-PsaD2*, At4g02770; *subunit VI*, At3g16140; *subunit X,* At1g30380; *PSI-E,* At4g28750), PSII (*6.1 kDa protein,* At2g30570), and the oxygen evolving complex (*23 kDa polypeptide,* At1g06680; *33 kDa polypeptide*, At5g66570), as well as components of the light harvesting system (At1g15810 and At5g54270).

In addition, the abundance of transcripts encoding the CP12 protein (At3g62410) and glucose 6-phosphate dehydrogenase (At5g40760) was increased, whereas ascorbate mediated decreases in mRNA encoding pyruvate kinase (At5g52920), phosphoribulokinase (At1g32060), starch synthase (At4g18240), phosphoenolpyruvate carboxylase (At1g53310), fructose-1, 6-bisphosphatase (At3g54050), and sedoheptulose-1,7-bisphosphatase (At3g55800) (Fig. 4B).

DISCUSSION

Ascorbate is a major redox buffer in all the compartments of the plant cell (19). It is therefore not surprising that ascor-

TABLE 3. MICROARRAY ANALYSIS OF ANTIOXIDANT TRANSCRIPTION PROFILES IN THE *ARABIDOPSIS VTC1* MUTANT AFTER INCUBATION IN 10 m*M* ASCORBATE

Transcript name	*Gene model*	*Probe set*	*Fold change*
Putative monodehydroascorbate reductase (NADH)	At3g52880	10119.htm	NC
Putative monodehydroascorbate reductase	At1g63940	1012.htm	NC
Putative monodehydroascorbate reductase (NADH)	At5g03630	1392.htm	NC
Putative monodehydroascorbate reductase (NADH)	At3g09940	1731.htm	NC
Partial GSH-dependent dehydroascorbate reductase 1	At1g19570	7936.htm	−2.04
Partial GSH-dependent dehydroascorbate reductase 1	At1g75270	9858.htm	NC
Stromal ascorbate peroxidase	At4g08390	12139.htm	NC
L-Ascorbate peroxidase-like protein	At4g32320	231.htm	NC
L-Ascorbate peroxidase (APX1)	At1g07890	3006.htm/4703.htm/7875.htm	NC
L-Ascorbate peroxidase (APX2)	At3g09640	NP	NP
L-Ascorbate peroxidase (APX3)	At4g35000	6299.htm	NC
Thylakoid-bound ascorbate peroxidase	At1g77490	6939.htm	NC
Putative L-ascorbate oxidase	At4g39830	3118.htm	NC
Cytosolic glutathione reductase	At3g24170	8235.htm	NC
γ-Glutamylcysteine synthetase	At4g23100	5019.htm	NC
Putative glutathione peroxidase	At2g43350	11434.htm	NC
Putative copper, zinc superoxide dismutase	At2g28190	7893.htm	NC
Superoxide dismutase (EC 1.15.1.1) (Fe) (fragment)	At4g25100	6323.htm	+2.66
Putative copper, zinc superoxide dismutase	At5g18100	14380.htm	+2.07
Superoxide dismutase	At1g08830	13422.htm	NC
Putative (Mn) superoxide dismutase	At3g10920	13406.htm	NC
Catalase 1 (CAT1)	At1g20630	11869.htm	NC
Catalase 2 (CAT2)	At4g35090	12472.htm/14385.htm/14750.htm/704.htm/7681.htm	NC
Catalase 3 (CAT3)	At1g20620	14784.htm	NC

Microarray analysis of antioxidant transcript profiles in the *Arabidopsis vtc1* mutant after incubation in 10 m*M* ascorbate was performed. Gene models and probe sets are as described in the Stanford Microarray Database (http://genome-www5.stanford.edu/MicroArray/SMD/). NC, no change; NP, not present.

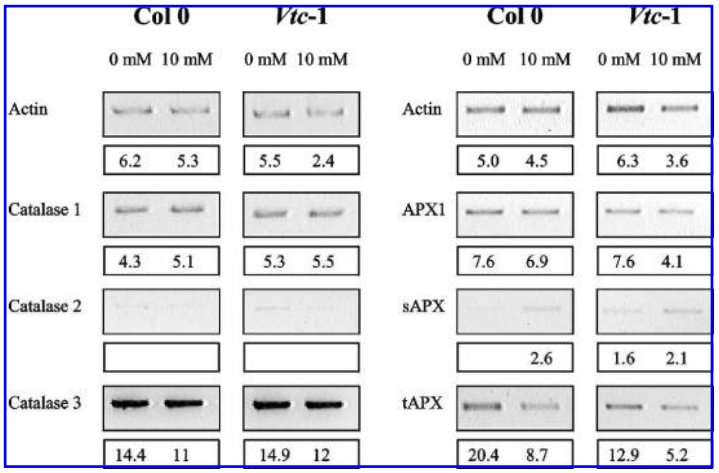

FIG. 3. Ascorbate-dependent changes in the expression of catalase and ascorbate peroxidase (APX) isoform transcripts.

bate availability modulates global gene expression. We have previously shown that plants are able to sense ascorbate deficiency and make appropriate changes in gene expression to modify the intracellular distribution of antioxidative enzymes (25) and activate pathways of defense that are normally associated with pathogen attack (21). Ascorbate deficiency modulates hormonal signaling pathways that impact on growth (21). These adjustments are a prerequisite for survival in response to depletion of tissue antioxidant capacity.

In the present study, we have explored the hypothesis that tissue ascorbate is involved in metabolite "cross-talk" between pathways comprising redox reactions. The data would suggest that the leaf ascorbate content is sensed and that this information is transduced in such a manner as to coordinate the activity of defense networks complementary to the antioxidant system. The glutathione pool is not changed in the *vtc1* mutant compared with the wild type (25) and appears to have little or no role in the signal transduction processes described here. The expression of key PR defense genes (*PR-1*, *PR-2*, and *PR-5*) is constitutively up-regulated in *vtc1,* whereas transcripts encoding antioxidative enzymes are largely unaffected by ascorbate depletion. When ascorbate is increased in *vtc1* leaves, only *PR-1* is decreased. In this case, transcripts encoding dehydroascorbate reductase and peroxiredoxin are also decreased. Down-regulation of dehydroascorbate reductase transcripts is logical at times when ascorbate is abundant and the pool is largely reduced. Moreover, the expression of peroxiredoxins, which remove lipid peroxides, is modulated by ascorbate (9). The finding that ascorbate increases transcripts encoding metallothionein and glutathione transferases is interesting and, like the effects on PR proteins, confirms that ascorbate is involved in metabolic cross-talk between pathways of defense against oxidative stress and those conferring tolerance to pathogens and to xenobiotics. When ascorbate is abundant, it appears that xenobiotic detoxification pathways are enhanced.

The *vtc1* mutant shows no symptoms of increased oxidative stress and is essentially acclimated to a low ascorbate existence (25). Gene expression in *vtc1* is therefore constitutively modified with respect to the wild type (21). When *vtc1* mutant leaf discs were supplied with ascorbate, the tissue ascorbate content rapidly increased, but the ascorbate redox

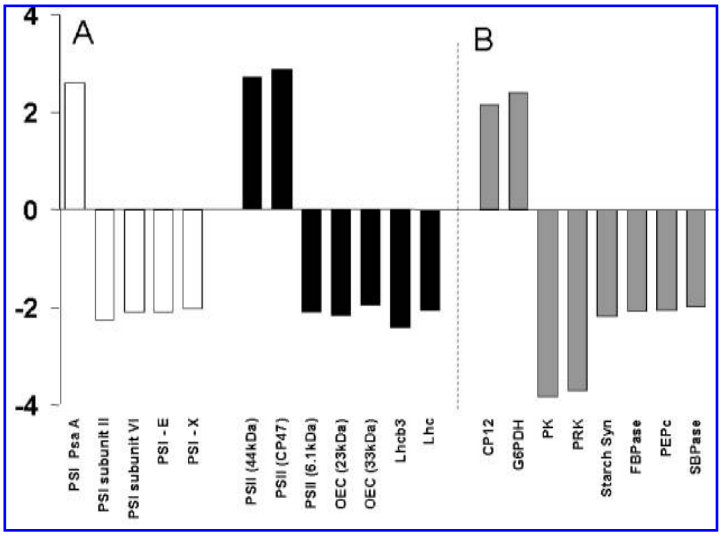

FIG. 4. High ascorbate-dependent modulation of the abundance of transcripts involved in photosynthetic electron transport (A) and photosynthetic carbon metabolism (B). CP, chlorophyll protein; FBPase, fructose-1,6-bisphosphatase; G6PDH, glucose 6-phosphate dehydrogenase; Lhc, light harvesting chlorophyll protein; OEC, oxygen evolving complex; PEPc, phospho*enol*pyruvate carboxylase; PK, pyruvate kinase; PRK, phosphoribulokinase; PSI, photosystem I; PSII, photosystem II; SBPase, sedoheptulose-1,7-bisphosphatase; Sid, salicylate induction-deficient; Syn, synthase.

state was not changed. Samples were taken at one time point only after the onset of feeding, and the array and RT-PCR data therefore provide only a single snapshot of the readjustments in gene expression that are induced by enhanced ascorbate. At the point of harvest, the ascorbate content of the *vtc1* leaf samples was enhanced by 14-fold compared with samples supplied with buffer alone. This rapid change in antioxidant availability caused a marked effect on genes associated with photosynthetic electron transport, which is the source of reducing power that maintains the ascorbate pool in the reduced state. As pointed out by Pfannschmidt *et al.* (22), redox chemistry involving the transfer of electrons or hydrogen atoms is central to energy conversion in photosynthesis. As ascorbate is the major redox buffer of the chloroplast lumen and cytosol, it is not surprising therefore that its availability modulates the transcription of photosynthetic genes. However, in contrast to genes encoded in the nucleus, which are repressed, those encoded on the chloroplast genome are activated.

The redox state of the intersystem electron transport carrier, plastoquinone, modulates the transcription of genes encoding reaction center apoproteins in such a way as to control the stoichiometry between the two photosystems (22). Given the information provided here, we can add to the general rules governing the redox signal-transduction pathway. The redox state of the ascorbate system, consisting of ascorbate-monodehydroascorbate and dehydroascorbate, was not changed by increasing the ascorbate supply, but the buffering capacity of the system was greatly enhanced. Pfannschmidt *et al.* (22) showed that the redox signal-transduction pathway can be short, the response time rapid, and the control direct. Here we show that, at least in the short term, large changes in the total amount of reductant available for the transfer of electrons or hydrogen atoms also modulate photosynthetic genes, but this acts in an antagonistic manner in the chloroplast and cytosol. The increase in ascorbate may have perturbed the coordination between chloroplast and nuclear gene expression.

The observed ascorbate-mediated decreases in transcripts encoding enzymes involved in the regenerative phase of the Benson–Calvin cycle (fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and phosphoribulokinase) provide another interesting insight into the readjustment of metabolism as reductant capacity is increased. These enzymes all require another reductant, reduced thioredoxin, for activation. Ascorbate is not generally considered to be involved in the thioredoxin-mediated activation of enzymes, but high ascorbate leads to repression of the transcription of these enzymes. Decreased transcript abundance may be important in counteracting the effects of high ascorbate, which might tend to favor increased activation. Moreover, it is interesting to note that transcripts encoding another thioredoxin-modulated enzyme, glucose 6-phosphate dehydrogenase, were increased by ascorbate. In the latter case, the enzyme is inactivated by reduced thioredoxin. Hence, there appears to be an inverse relationship between regulation of enzyme activity by thioredoxin and the changes in transcript abundance triggered by variations in ascorbate. Transcripts of Benson–Calvin cycle enzymes that are activated by reduced thioredoxin are repressed by high ascorbate. Conversely, transcripts of enzymes such as glucose 6-phosphate dehydrogenase that are inactivated by reduced thioredoxin are induced by high ascorbate. These observations may indicate a redox-mediated compensation mechanism between transcription and posttranslational regulation. It is interesting to note that transcripts encoding another redox-modulated chloroplast component, CP12, whose activity is regulated by NADPH availability, were increased in the presence of high ascorbate. Pyruvate kinase and phosphoenolpyruvate carboxylase transcripts were also decreased, suggesting that the transcription of these enzymes is also sensitive to the availability of reductant.

In the longer term, the redox system must adjust to the presence of high ascorbate as ascorbic acid is one of the most abundant compounds in green leaves, reaching concentrations as high as 100–500 m*M* in extreme environments (7, 19). In favorable conditions, it represents 10% of the total soluble carbohydrate pool (19, 24). The pathway of ascorbate biosynthesis appears to be controlled by both developmental triggers and environmental cues (24). To date, there is no indication that there is a ceiling on the extent to which ascorbate can accumulate in leaves as in continuous light it is continuously synthesized and accumulated (23).

ABBREVIATIONS

APX, ascorbate peroxidase; CAT, catalase; Col, columbia; Eds, enhanced disease susceptibility; H_2O_2, hydrogen peroxide; PAL, phenylalanine ammonium lyase; PR, pathogenesis-related; PSI, photosystem I; PSII, photosystem II; RT-PCR, reverse transcriptase–polymerase chain reaction; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; Sid, salicylate induction-deficient; SOD, superoxide dismutase; sAPX, stromal ascorbate peroxidase; tAPX, thylakoid ascorbate peroxidase; Vtc, vitamin C.

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