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Leaf Vitamin C Contents Modulate Plant Defense Transcripts and Regulate Genes That Control Development through Hormone Signaling[W]

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Vitamin C deficiency in the Arabidopsis mutant *vtc1* causes slow growth and late flowering. This is not attributable to changes in photosynthesis or increased oxidative stress. We have used the *vtc1* mutant to provide a molecular signature for vitamin C deficiency in plants. Using statistical analysis, we show that 171 genes are expressed differentially in *vtc1* compared with the wild type. Many defense genes are activated, particularly those that encode pathogenesis-related proteins. Furthermore, transcript changes indicate that growth and development are constrained in *vtc1* by the modulation of abscisic acid signaling. Abscisic acid contents are significantly higher in *vtc1* than in the wild type. Key features of the molecular signature of ascorbate deficiency can be reversed by incubating *vtc1* leaf discs in ascorbate. This finding provides evidence that many of the observed effects on transcript abundance in *vtc1* result from ascorbate deficiency. Hence, through modifying gene expression, vitamin C contents not only act to regulate defense and survival but also act via phytohormones to modulate plant growth under optimal conditions.

INTRODUCTION

L-Ascorbic acid (vitamin C) is a multifunctional compound in both plants and animals. This metabolite is one of the most abundant in green leaves. In favorable conditions it represents 10% of the total soluble carbohydrate pool (Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). A plant-specific pathway of vitamin C biosynthesis has been described and appears to be controlled by both developmental triggers and environmental cues (Smirnoff and Wheeler, 2000). Much attention has focused on the antioxidant role of vitamin C, but in both plants and animals, this vitamin also is important as a cofactor for a large number of key enzymes (Arrigoni and de Tullio, 2000). Furthermore, vitamin C influences mitosis and cell growth in plants, although mechanistic details are lacking (Noctor and Foyer, 1998; Arrigoni and de Tullio, 2000; Smirnoff and Wheeler, 2000).

To study the impact of modified endogenous vitamin C content without the potentially obscuring effects of concomitant changes in redox state, we exploited the availability of an Arabidopsis mutant, *vtc1* (Conklin et al., 1996). This mutant has constitutively low vitamin C content as a result of impaired biosynthesis. A point mutation at position +64 of the GDP-mannose pyrophosphorylase (GMPase) gene sequence encodes a Pro-to-Ser change at position 22 of the translated sequence, resulting in substantially decreased GMPase activity, even though transcript abundance is not affected (Conklin et al., 1996, 1999). Leaf vitamin C contents are 70% lower than in the wild type (Conklin et al., 1996; Veljovic-Jovanovic et al., 2001). Vitamin C is the major antioxidant of the leaf apoplast but is absent from the apoplast of *vtc1* leaves (Veljovic-Jovanovic et al., 2001). There appears to be little compensation for decreases in leaf ascorbate by increases in other antioxidants (Veljovic-Jovanovic et al., 2001), except for nonspecific guaiacol-type peroxidases. Ascorbate-mediated changes in the intracellular distribution of antioxidant enzymes have been described, but the overall capacity of the antioxidant system is largely unchanged, except for a marked increase in nonspecific peroxidase activity (Conklin et al., 1996, 1999; Veljovic-Jovanovic et al., 2001).

Unperturbed overall leaf antioxidant capacity is indicated further by leaf H_2O_2 contents in the mutant, which are similar to those in the wild-type (Veljovic-Jovanovic et al., 2001). Despite this fact, *vtc1* is smaller than the wild-type plant and shows retarded flowering and accelerated senescence (Veljovic-Jovanovic et al., 2001). Because *vtc1* does not accumulate H_2O_2 and the redox states of leaf antioxidants are not changed (Conklin et al., 1996; Veljovic-Jovanovic et al., 2001), these phenotypic effects are linked to modified amounts of vitamin C rather than to a general change in cellular redox balance. Although vitamin C contents are decreased markedly in *vtc1*, the mutant still maintains a global leaf concentration of ~0.7 mM (Conklin et al., 1996; Veljovic-Jovanovic et al., 2001), which is equivalent to at least 4 mM vitamin C in the cytosol, because concentrations probably are very low in the large leaf cell vacuole. Thus, this mutant provides an excellent system in which to examine the effects of physiologically relevant decreases in vitamin C.

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Although Haughn and Somerville (1987) estimated that the number of ethyl methanesulfonate mutations present in any given M2 population is ~200 and the *vtc1* mutant line used in these studies has not been subjected to multiple backcrosses to wild-type plants, all published work on this and similar mutants has the same limitation. Therefore, it remains a formal, if very minor, possibility that the delayed flowering and smaller size of the *vtc1* plants is attributable not to the ascorbate deficiency but to another mutation in the mutant background. However, to date, the smaller size/delayed flowering has been found to cosegregate with the ascorbic acid deficiency. Moreover, it is clear that altered transcriptional abundance is linked to ascorbate deficiency, because feeding this metabolite reverses the deficiency signature. These observations lead us to conclude that changes in vitamin C content influence plant growth and development by modulating the expression of specific suites of genes involved in defense and hormonal signaling pathways.

RESULTS AND DISCUSSION

We analyzed the link between low vitamin C and the *vtc1* phenotype through an integrated approach involving transcriptome analysis, physiological measurements, and biochemical assays. Initially, the transcriptome data obtained were grouped as five separate pair-wise comparisons and analyzed using the Affymetrix Gene Expression Analysis Software GeneChip version 3.3. This preliminary analysis indicated that 20 genes were detected as differentially expressed in all five pair-wise comparisons (i.e., they were increased or decreased systematically in all five chips) (Figure 1). However, the total number of differentially expressed genes varied significantly among the different array pairs as a result of background noise and variation between samples. Therefore, we validated and extended the primary analysis by reassembling and reanalyzing the data to treat all replicate analyses as a single experiment using the DNA Chip Analyzer (dChip). After sorting, the expression levels across all of the arrays were normalized against the highest median overall mean intensity level for one plate.

In the dChip analysis, "rogue" probe responses that fell outside the pattern found on other plates were identified using the calculations described by Li and Wong (2001). Baseline expression was set to exclude values falling within 20% of median probe intensities. Expression data for genes falling above or below this baseline are shown in Figure 2. Using this statistical analysis, we were able to identify with confidence changes in gene expression that could not have been revealed by simple pair-wise comparisons. The genes with the highest levels of expression were clustered using dChip to compare expression across plates (Figure 3). Of 8300 transcripts detected by the chip, 171 exhibited significantly differential expression between Columbia (Col0) and *vtc1* (see supplemental data online).

Of the 171 transcripts showing differential expression in the mutant, 138 represented genes either of known function or to which function could be assigned putatively on the basis of homology. Of these, 54 (32%) encoded DNA binding proteins (21 genes) or proteins implicated in cell cycle control (12 genes), signaling (9 genes), and developmental (12 genes) processes (Figure 4). These included transcripts for kinase and kinase receptors as well as for MYB factors and zinc finger proteins (see supplemental data online). Transcript levels for 42 proteins involved in metabolism were modified (see supplemental data online), 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). Altered changes in transcript abundance described hereafter were confirmed by reverse tran-

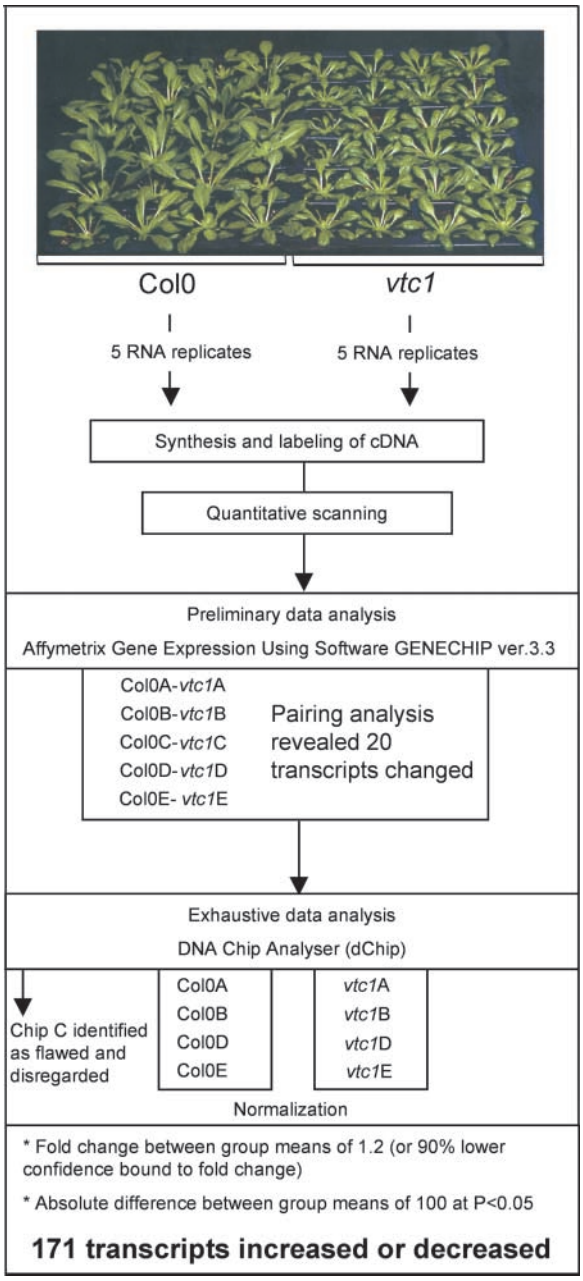

Figure 1. Strategy for Microarray Analysis of Gene Expression in the Vitamin C–Deficient *vtc1* Arabidopsis Mutant.

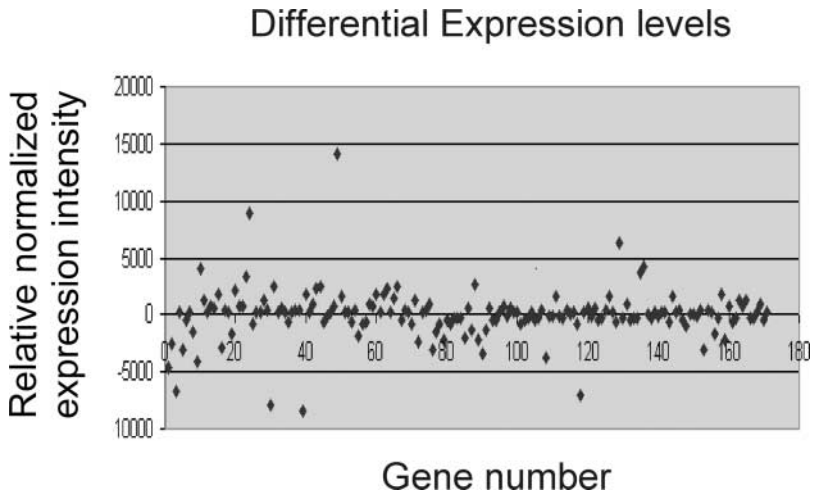

Figure 2. Scatterplot of the 171 Transcripts That Show Consistent Differential Abundance in Col0 and *vtc1*.

The Col0 and *vtc1* arrays were normalized against the expression levels of the array with the median overall intensity (sample Col0A had a median overall intensity of 1017). The model-based expression levels were calculated with the dChip package according to Li and Wong (2001). Col0 then was compared with *vtc1*, selecting expressed/baseline $>$ 1.2 and baseline/expressed $>$ 1.2, taking the lower 90% confidence boundary of fold change. Additionally, expression levels showing a difference from the baseline of $\leq$100 (10% of median) were rejected. The scatterplot illustrates the normalized intensities for the genes selected with the criteria described above. The *y* axis shows expressed minus baseline intensities, with values above the *x* axis indicating genes with greater intensity in *vtc1* and values below the line indicating genes with less intensity in *vtc1*. The gene number is arbitrary, according to the Affymetrix spot list. It is not intended to be related to the gene order in the supplemental data online but to give a precise indication of differences in expression levels.

scriptase–mediated PCR; examples of these results are shown in Figure 5. In this analysis, we emphasize the major features of the vitamin C deficiency signature.

Regulation of Photosynthesis

Among genes involved directly in photosynthesis, the only significant change observed was in *rbcL* transcripts encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which were decreased by ~20% in the *vtc1* mutant (Table 1). Biochemical analysis showed that this decrease was similar to those in the amounts of Rubisco protein and maximal activities (Table 1). The activation state of the enzyme, however, was higher in *vtc1* (Table 1), explaining the unaffected rates of photosynthetic CO_2 fixation (Veljovic-Jovanovic et al., 2001). Vitamin C plays several roles in photosynthetic energy partitioning. We showed previously that the capacity for photosynthetic thermal energy dissipation, which involves an enzyme that uses vitamin C as a cofactor, is not affected appreciably in *vtc1* except under very high light (Veljovic-Jovanovic et al., 2001). However, at high irradiances, well in excess of those that saturate photosynthesis in Arabidopsis, *vtc1* leaves show decreased nonphotochemical quenching (NPQ) values (Veljovic-Jovanovic et al., 2001). Moreover, this view is supported by the results of a recent study on another ascorbate-deficient Arabidopsis mutant, *vtc2*. This mutant has 25% of the wild-type leaf ascorbate contents and shows lower NPQ values than Col0 at irradiances of $>$400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Muller-Moule et al., 2002). It should be noted that these are photoinhibitory irradiances in Arabidopsis. At optimal growth irradiances (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for Arabidopsis, both *vtc2* and Col0 had similar, very low levels of NPQ (Muller-Moule et al., 2002).

These observations demonstrate that NPQ is not limited by vitamin C availability at typical growth irradiances for this species. Therefore, we conclude that NPQ in both *vtc* mutants and in the Col0 control generally is low and that it is not significantly different between the genotypes under optimal growth conditions. Nevertheless, it is tempting to speculate that limitations on the violaxanthin deepoxidase reaction may have strategic relevance under high light. Because violaxanthin is a precursor of neoxanthin, its accumulation under high light may drive abscisic acid (ABA) synthesis and hence favor other defense responses.

In addition to CO_2 fixation, C_3 plants such as Arabidopsis have several other important sinks for photosynthetic energy. Chief among these are photorespiration and the Mehler peroxidase reaction, which depends on vitamin C (Noctor and Foyer, 1998; Foyer and Noctor, 2000; Noctor et al., 2002). Both of these processes are important alternative sinks that protect the leaf from deleterious processes such as photoinhibition and photooxidation (Foyer and Noctor, 2000). It is conceivable that a compromised capacity for energy use could provoke the developmental changes observed in *vtc1*. Therefore, we determined whether the lower leaf vitamin C contents in the mutant affect the Mehler peroxidase reaction. The total rate of electron flow from chlorophyll fluorescence analysis (J_{II}) was compared with the flow accounted for by carbon fixation and photorespiration (J_e). These two parameters showed a curvilinear relationship

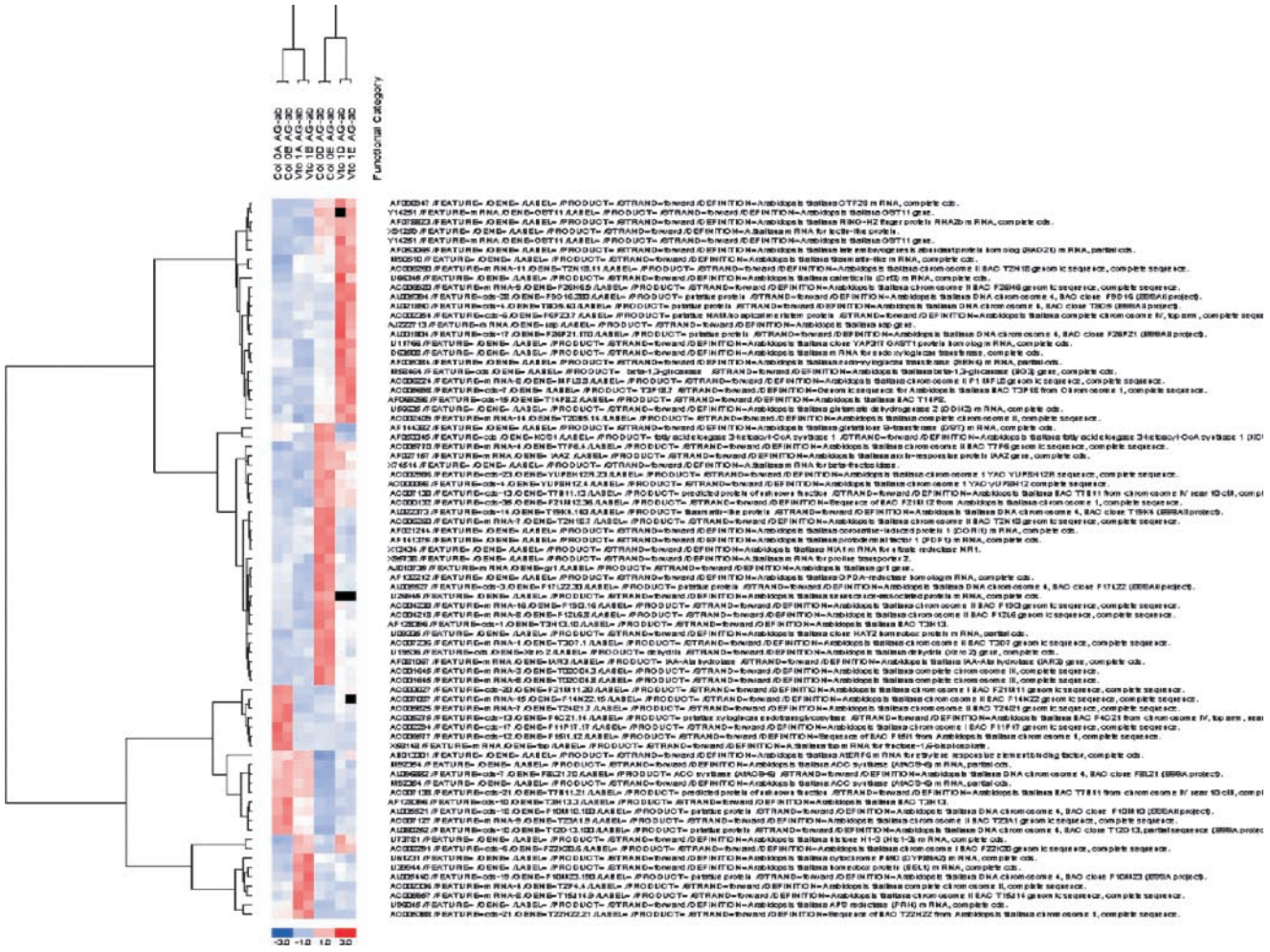

Figure 3. Part of the Hierarchical Clustering of the Transcripts That Showed Significant Changes in Abundance between *vtc1* and the Wild Type (Col0).

Using the dChip package, the following criteria were selected to show a representative clustering of the data. Data were normalized as for the scatterplot (Figure 2), but the difference between expression level and baseline was increased to 200 (20% of median levels) to illustrate the more strongly exhibited features. Outliers as determined by Li and Wong (2001) were treated as missing data (these show as black on the cluster diagram). Further filtering was applied to the data. The variation across sample was restricted by setting $0.5 <$ standard deviation/mean < 2. Spots exhibiting >50% probability were included. The expression level was >500 in all samples. Degrees of red and blue indicate the extent of positive and negative fold change, respectively. Hierarchical clustering is considered by many as a means of visualization. For simplicity, the relative changes between Col0A and *vtc1A*, et cetera, could be compared. The tree branches represent a hierarchical organization of expression levels. It should be noted that the clustering is a statistical tool, and it is doubtful if it has any biological significance. It essentially shows similar expression levels and groups these clumps of expression levels together. For reasons of space, the clustering shows a restricted number (approximately half) of the 171 transcripts of interest.

that was similar in both types of plant (Figure 6). Comparison of J_{II} with J_e allows the estimation of electron flow associated with other processes, such as the vitamin C–dependent Mehler peroxidase reaction. The parameter $J_{II} - J_e$ tended to increase with irradiance but was not significantly different between the two plant types (Figure 6). These results suggest that the comparatively low concentrations of ascorbate in the mutant are sufficient to maintain optimal rates of the Mehler peroxidase reaction (Foyer and Noctor, 2000). Hence, the changes in growth and flowering linked to vitamin C content are not the result of any perturbation of photosynthetic capacity or function.

Induction of Defense Proteins

The most striking changes in transcript abundance were observed for genes involved in responses to biotic stress. In par-

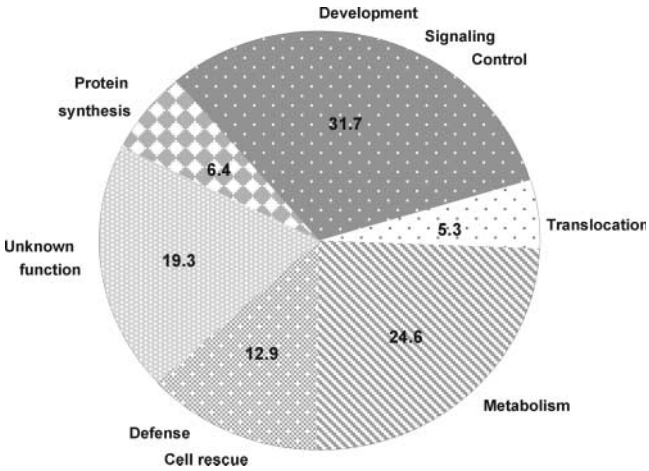

Figure 4. Low Vitamin C Modifies Gene Expression in Arabidopsis.

Microarray analysis was used to compare leaf transcript abundance in the vitamin C–deficient Arabidopsis mutant *vtc1* and the corresponding wild type, Col0. A total of 171 transcripts were significantly and reproducibly modified in abundance and assigned to functional categories. The chart shows the distribution between functional classes of transcripts that differ in abundance in *vtc1* and the wild type. Numbers indicate percentages of total transcripts for which significant differences in abundance were observed. For further details, see the supplemental data online.

ticular, transcripts encoding pathogenesis-related (PR) proteins and other lytic enzymes were increased (Figure 7). Early events during incompatible plant–pathogen interactions include an oxidative burst and strong induction of Phe ammonia-lyase (PAL) (Lamb and Dixon, 1997). PAL activity is required for both the production of antimicrobial phytoalexins and the synthesis of salicylic acid, which induces PR proteins and elicits systemic acquired resistance to the pathogen (Lamb and Dixon, 1997). In *vtc1*, however, PR proteins and other defensive enzymes were upregulated constitutively, whereas transcripts of three PAL genes were not significantly different from wild-type transcripts (Figure 7). This finding is in contrast to the elicitation of systemic acquired resistance–associated transcripts by H_2O_2 treatment of Arabidopsis, which is accompanied by the induction of PAL (Desikan et al., 2001). Moreover, we showed previously that overall leaf H_2O_2 contents are similar in the mutant and the wild type (Veljovic-Jovanovic et al., 2001). It is noteworthy that the Arabidopsis mutant *sid*, which does not accumulate salicylic acid, nevertheless shows induction of PR proteins in response to infection (Nawrath and Metraux, 1999).

The *vtc1* mutant was isolated by means of its hypersensitivity to atmospheric ozone (Conklin et al., 1996). Ozone treatment is known to induce PR proteins, and this is thought to occur through the activation of signaling elements involved in the pathogen response, such as the accumulation of active oxygen species and the ensuing synthesis of salicylic acid (Kangasjarvi et al., 1994). This view is supported by the coordinated expression of PR1 and PAL in poplar exposed to ozone (Koch et al., 1998). The absence of PAL upregulation in *vtc1* shows that low vitamin C is associated with the induction of PR proteins through signaling pathways that are independent of active oxygen species and salicylic acid. The mutant's hypersensitivity to ozone (Conklin et al., 1996) may be the result of a potentiating effect of low vitamin C on defense induction, which thereby more readily promotes cell death. Thus, plant nutritional status and environmental factors that modulate vitamin C contents may influence disease susceptibility.

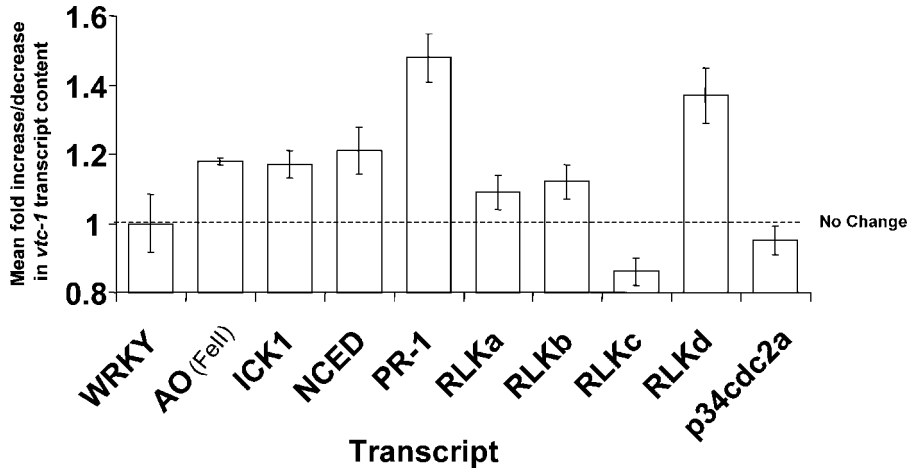

Figure 5. Validation of Results from the Microarray Analysis.

The transcript abundance of a selected range of genes whose expression was altered significantly in the microarray experiment was analyzed by reverse transcriptase–mediated PCR. The analysis was performed using primers specific to the following genes: WRKY binding protein (WRKY; At4g923180), ascorbate oxidase (AO[FeII]; At4g10500), cyclin-dependent kinase (ICK1; At2g23430), 9-*cis*-epoxicarotenoid dioxygenase (NCED; At4g19170), pathogenesis-related protein1 (PR1; At2g14610), receptor-like kinase a (RLKa; At4g23260), receptor-like kinase b (RLKb; At4g23150), receptor-like kinase c (RLKc; At5g35370), receptor-like kinase d (RLKd; At4g04500), and p34 cyclin-dependent kinase 2a (p34cdc2a; X57839).

Table 1. Rubisco Transcripts, Protein, and Activity in *vtc1* and the Wild Type, Col 0

Plant Type	*rbcL* Transcripts (%)	Total Protein (Relative Intensity)	Maximal Activity (nmol·mg^{-1} protein·min^{-1})	Activation State (%)
Col0	100	3.6 ± 0.3	328 ± 111	74 ± 4
vtc1	77	3.1 ± 0.3	230 ± 69	82 ± 3

Transcripts encoding the large subunit (*rbcL*) were quantified using microchip technology.

Regulation of Growth by Modulation of Hormone Metabolism

Plant growth and development are controlled by phytohormones such as auxins, gibberellins, and ABA. The slow growth and retarded flowering of the *vtc1* mutant (Veljovic-Jovanovic et al., 2001) support a role for vitamin C in the control of plant growth. As shown above, disruption of photosynthetic capacity or regulation can be discounted as the primary cause of these characteristics (Figure 6). The abundance of a number of ABA-modulated transcripts was increased in *vtc1* compared with Col0 (Figure 8). Thus, we measured the ABA content of *vtc1* and Col0 leaves. The ABA contents of *vtc1* leaves were 60% greater than those measured in Col0 (Figure 8).

ABA is essentially a cell survival hormone that induces metabolic arrest and sustains stress resistance. It promotes dormancy in seeds, downregulates photosynthetic carbon assimilation by closing stomata when water is scarce, and mediates adaptive changes to environmental cues, especially drought stress (Himmelbach et al., 1998; Bianco and Dalstein, 1999). Tissues that accumulate ABA in response to stress no longer grow as a result of reduced cell elongation and mitotic activity. Biochemical studies and analyses of ABA-deficient mutants indicate that one important route to ABA is through oxidative cleavage of neoxanthin to xanthoxin (Milborrow, 2001) catalyzed by NCED. This enzyme is dependent on vitamin C for activity (Arrigoni and de Tullio, 2000) and is known to regulate ABA biosynthesis in response to drought stress (Qin and Zeevaart, 1999; Thompson et al., 2000). Because vitamin C is low in *vtc1*, we predict that the flux through the dioxygenase reaction may be restricted accordingly. In such circumstances, the amount of enzyme may be increased in an attempt to compensate for limitations in flux. We presume that this is the case with the NCED reaction and that transcripts encoding this enzyme are increased in these circumstances to increase maximal catalytic capacity.

Consistent with the increases in ABA content, the transcript analysis provides evidence of the upregulation of ABA synthesis and consequent signaling when vitamin C is low. First, NCED transcripts were increased in *vtc1* compared with Col0 (Figure 8B). Second, two pectin methylesterase (At1g53830 and At1g53840) messages were repressed. The extractable activity of this enzyme is known to be decreased by ABA treatment of germinating seeds (Micheli, 2001). Third, phosphoribosyl anthranilate transferase transcripts were increased in *vtc1*. Phosphoribosyl anthranilate transferase is a key enzyme in Trp synthesis. This observation is consistent with the observed accumulation of Trp in barley leaves treated with ABA (Ogura et al., 2001). Fourth, transcripts were induced for the ABA-responsive dehydrin RAB18 (Welin et al., 1994). Two more transcripts induced in *vtc1* are known to encode (At4g15910) or are likely to encode (At4g02200) dehydrins (Figure 8B). Also, HAT5 and BEL1, which are homologues of ABT5, are increased in *vtc1* (Figure 9). Fifth, the ABA-mediated regulation of stomata involves the modification of ion channel activities at the plasmalemma and tonoplast (MacRobbie, 1995), and a tonoplast-intrinsic protein (aquaporin; At2g36830) involved in cell expansion was repressed in *vtc1* (see supplemental data online). Sixth, transcripts belonging to heat-shock protein families known to be responsive to drought (Ingram and Bartels, 1996) were induced in *vtc1* (HSP70 and HSP81; Figure 8B). Seventh, low vitamin C was associated with the induction of a histone linker transcript (*Hsp3-1*) that belongs to a family of histone genes known to be responsive to drought (Ascenzi and Gantt, 1997).

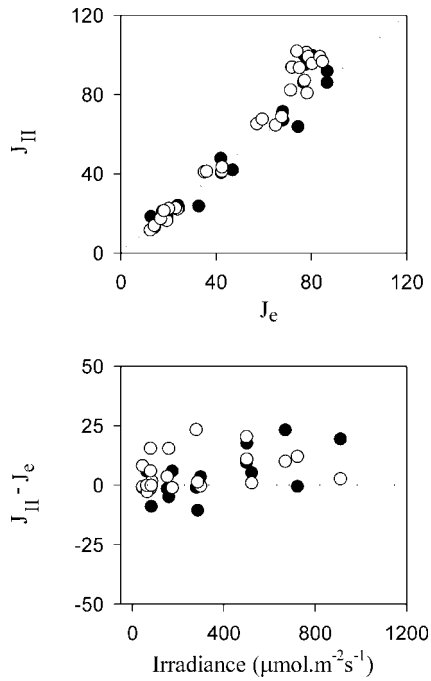

Figure 6. Relationship between Total Photosynthetic Electron Flux (J_{II}) and Flux Linked to Ribulose-1,5-Bisphosphate Carboxylation and Oxygenation (J_e) in Attached Leaves of Arabidopsis Illuminated at Different Irradiances in Air.

Open circles, wild type (Col0); closed circles, *vtc1*.

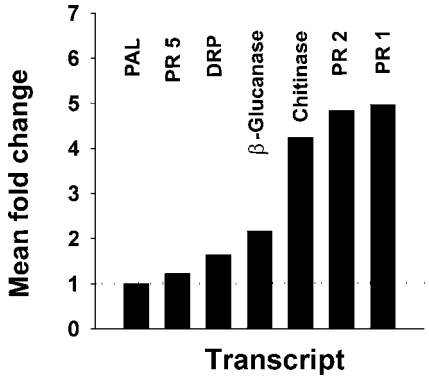

Figure 7. Induction of Genes Involved in Pathogen Resistance in *vtc1*.

Three PAL sequences were present on the microarray chip, PAL1 (accession number X84728), PAL2 (At3g53260), and PAL3 (At5g04230); as well as pathogenesis-related proteins 1 (PR1; At2g14610), 2 (PR2; At3g57260), and 5 (PR5; At1g75040); a putative disease resistance protein (DRP; At4g13900); β-glucanase (At4g16260); and chitinase (At2g43570). Col0 and *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 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance, 60% [v/v] RH, and day/night temperatures of 23 and 18°C). 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).

Finally, a cyclin-dependent kinase inhibitor, ICK1 (At2g234330), from Arabidopsis was induced in *vtc1* (Figure 8B). ICK1 is a member of the cyclin-dependent kinase inhibitor (kinesin-regulatory protein or kinesin-inhibitor protein–related protein) family. It is induced by ABA treatment, leading to the inhibition of cell division and plant growth (Wang et al., 2000). Overexpression of this gene has been shown to reduce cell division and organ size in Arabidopsis (Mizukami, 2001).

Together, these observations suggest that ABA accumulation results from ascorbate deficiency and hence triggers ABA signaling events and associated processes. Therefore, ABA-dependent pathways transduce information concerning leaf vitamin C contents. Leaf ABA was increased in the mutant by 1.6-fold (Figure 8C). Modulation of the hormonal control of plant development by vitamin C content is indicated further by the upregulation of an ethylene-responsive transcription factor in *vtc1* (see supplemental data online). Both ABA and ethylene are stress-linked hormones that inhibit stem elongation in terrestrial plants, acting antagonistically to auxins and gibberellins (Kende et al., 1998). Gibberellins and ABA also act antagonistically in controlling the remobilization of seed reserves (Gómez-Cadenas et al., 2001), and gibberellins play an important role in the promotion of flowering (Blázquez et al., 1998). Therefore, ABA signaling is responsible at least in part for the dwarf late-flowering phenotype of *vtc1*.

In addition, disruption of gibberellin homeostasis and associated signaling was shown by the repression of transcripts for a microtubule-associated protein and the induction of a gene putatively encoding an enzyme involved in gibberellin synthesis (see supplemental data online). Possible interactions exist between the pathway of ascorbate synthesis and gibberellin signaling. The SPINDLY protein, known to be an *N*-acetylglucosamine transferase (Hartweck et al., 2002), is a negative regulator of gibberellin signaling (Olszewski et al., 2002). An additional mechanism linking ascorbate content and development is the requirement of ascorbate as a cofactor for enzymes involved in phytohormone synthesis (Arrigoni and de Tullio, 2000). NCED transcripts are upregulated in response to the ascorbate deficiency, perhaps as compensation for the decreased cofactor availability. Our data show the induction of two transcripts encoding 2-oxoglutarate–dependent dehydrogenases (At4g10500 and At2g36690), which may be involved in gibberellin synthesis and which, like NCED, use ascorbate as a cofactor. The balance between ABA and gibberellin contents, which could be one link between ascorbate concentration and growth rate, may depend on the extent to which enzymes are induced to compensate for the effects of ascorbate deficiency on catalysis through key steps.

There was no indication of changes in gene expression mediated by salicylic acid, but transcript levels for genes involved in indole metabolism suggest the possible modulation of auxin contents (see supplemental data online). For example, the observed increase in phosphoribosyl anthranilate transferase transcripts implies enhanced indoleacetic acid synthesis via increased Trp synthesis in *vtc1*. Enhanced leaf indoleacetic acid contents can cause dwarf phenotypes in transgenic plants. Therefore, it is possible that the increase of leaf indoleacetic acid could contribute to the observed phenotype of the mutant.

Reversal of Changes in Transcript Abundance by Ascorbate Feeding

The first array experiments were performed to determine transcriptome changes in the *vtc1* mutant, which is adapted to low endogenous ascorbate concentrations. To investigate the effect of ascorbate on gene transcription, we performed a second microarray comparison of *vtc1* leaf discs supplied with 10 mM 3-(*N*-morpholino)-propanesulfonic acid buffer, pH 6.0, in the absence or presence of 10 mM ascorbate for 16 h in darkness. As a result of this treatment, the ascorbate content of the *vtc1* leaves was increased 10-fold to values similar to those of Col0 treated in the same way. The ascorbate content in ascorbate-treated leaves of Col0 and *vtc1* leaf discs after 16 h of incubation was between 15 and 22 μmol/g fresh weight, the pool being reduced by >90% in all cases. The increase in leaf disc ascorbate resulted in a large number of changes in transcript abundance (see supplemental data online). The first set of arrays was performed using the Affymetrix chip system, and the second set used the Stanford arrays. The second analysis was less rigorous than the first in that only two chips were compared for *vtc1* plus and minus ascorbate. Therefore, the level of confidence is less for the second (1.9-fold) than for the first (1.2-fold) series of array analyses. Despite the differences in array systems used, similar coding sequences were found on both types of chip, and the trend in transcript abundance observed in *vtc1*

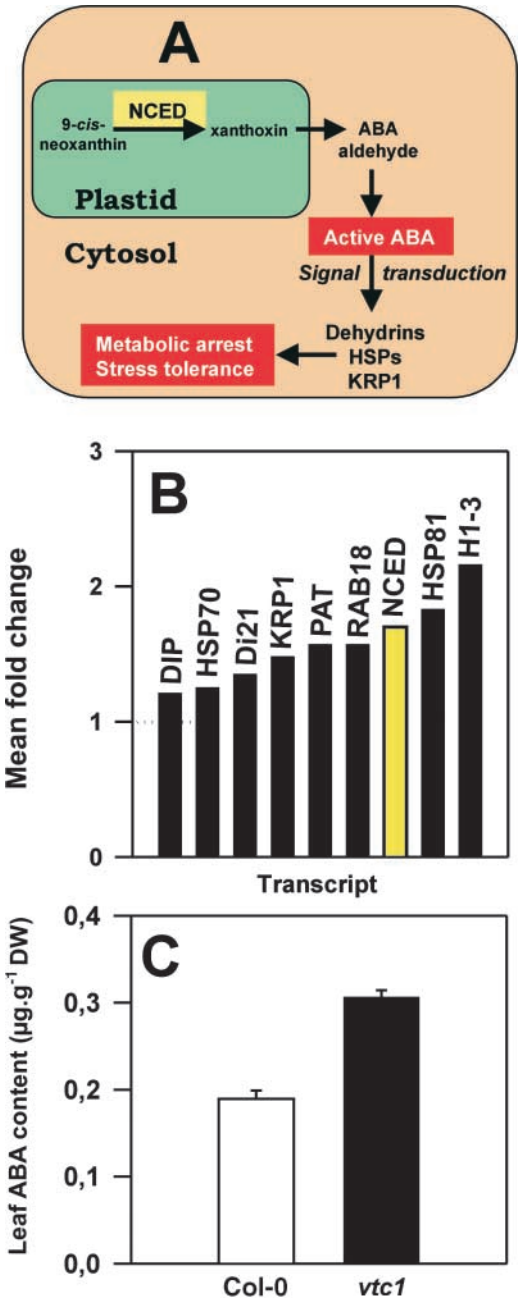

Figure 8. ABA Synthesis and Signaling Is Upregulated in the Vitamin C–Deficient Arabidopsis Mutant *vtc1*.

(A) Scheme showing the synthesis of ABA via chloroplastic NCED followed by the signaled shutdown of metabolism and cell division, accompanied by enhanced hardiness. Dehydrins, dehydration-induced proteins; HSPs, heat-shock proteins; KRP1, cyclin-dependent kinase inhibitor.

(B) Observed induction in *vtc1* of NCED transcripts (At4g19170; yellow bar) and transcripts responsive to ABA or to drought (black bars). DIP, putative drought-induced protein (At4g02200); HSP70, heat-shock protein70 (At3g12580); Di21, dehydrin 21 (At4g15910); KRP1, kinesin inhibitor protein–related protein; ICK1, a cyclin-dependent kinase inhibitor (At2g23430); PAT, phosphoribosyl anthranilate transferase (At4g00700); RAB18, responsive to abscisic acid protein 18 (At5g66400); HSP81, heat-shock protein81; H1-3, histone H1-3 (At2g18050).

(C) Increases in leaf ABA content in *vtc1*. Data are means ± SE of nine independent analyses. DW, dry weight.

was reversed by the ascorbate treatment. Key examples are shown in Figure 9.

Is the *vtc1* Phenotype Caused by Insufficient GMPase Activity for Cell Wall Biosynthesis or Deficiencies in Substrate Supply for Glycosylation?

Because the mutation in GMPase in *vtc1* affects an early step in the pathway, it could influence cell wall formation as well as ascorbate biosynthesis. Therefore, it is important to consider the possible effects of changes in the capacity of cell wall biosynthesis on the observed changes in transcript abundance reported here. There are two key issues that merit discussion in this regard: first, the effects of the mutation on the capacity for cell wall biosynthesis, which causes a decrease in cell wall mannose; second, effects related to possible changes in glycosylation capacity. It should be noted, however, that Conklin et al. (1999) and Keller et al. (1999) considered decreased ascorbate content a determining feature of the properties associated with low GMPase activity (e.g., ozone hypersensitivity). Antisense expression of GMPase in potato led to a decrease in the mannose content of the cell wall (30 to 50%), but no significant changes in soluble metabolites other than ascorbate (44 to 77% lower) were observed (Keller et al., 1999). The reduced mannose content of the wall of the GMPase antisense plants presumably was caused by the reduced formation of GDP-mannose. However, it is difficult to compare the phenotype observed in the study by Keller et al. (1999) with that presented here because they show different properties. GMPase antisense plants in culture had no phenotype for the first 10 weeks of growth and then entered early senescence. When grown in soil, the GMPase antisense plants were significantly smaller than the controls, as were *vtc1* plants. Unlike the GMPase antisense plants, however, the *vtc1* mutant was healthy throughout the life cycle, showing none of the evidence of oxidative stress found in the antisense GMPase phenotype (Keller et al., 1999).

It is not clear whether the severe phenotype observed in the GMPase antisense plants results from low cell wall mannose or low ascorbate. Much smaller decreases in mannose in the cell wall were found in *vtc1* compared with potato antisense plants (N. Smirnoff, personal communication), and this finding could explain why *vtc1* is much more healthy than the antisense GMPase potatoes. Nevertheless, it is important to address the issue of possible effects of changes in cell wall mannose content on gene expression. To date, there have been no reports of effects of mannose on gene transcription other than those associated with sugar metabolism, and signals arising from changes in cell wall composition have yet to be determined. Therefore,

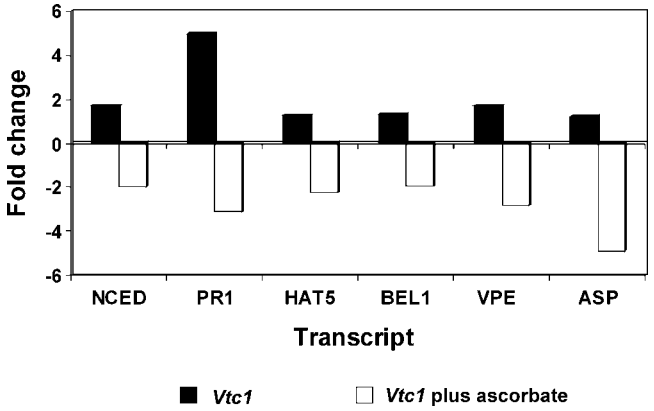

Figure 9. Reversion of Gene Expression in *vtc1* by Ascorbate Feeding.

Transcript abundance of a selected range of genes whose expression was reversed by ascorbate feeding in the Stanford microarray experiment. PR1, pathogenesis-related protein1; HAT5, homeobox Leu zipper transcription factor HAT5; BEL1, homeobox Leu zipper transcription factor BEL1; VPE1, vacuolar processing enzyme1; ASP, aspartyl protease. Values indicate positive and negative fold changes in *vtc1* and *vtc1* incubated in 10 mM ascorbate, respectively.

although we cannot exclude the possibility that some of the transcript changes are linked to cell wall metabolism, we favor the conclusion that the contribution of changes in cell wall mannose content to the observed modulation of gene expression is less important than that of ascorbate. This conclusion is supported by the effect of ascorbate feeding on the transcriptome (Figure 9).

vtc1 is a relatively weak mutation with minor effects on cell wall biosynthesis compared with the *cyt1* mutation in the same gene. In the *cyt1* mutant, a decrease in the availability of GDP-mannose leads to a deficiency in *N*-glycosylation capacity and a fivefold decrease in cellulose content (Lukowitz et al., 2001). The *cyt1* phenotype is very different from that of *vtc1*. *cyt1* is essentially lethal because it has a complete lack of GMPase activity. Because we do not know whether *N*-glycosylation capacity is changed in *vtc1*, it is not possible to completely discount the effects of decreased *N*-glycosylation on gene transcription. Moreover, it is conceivable that GDP-mannose could be limiting for *N*-glycosylation even if it is sufficient for cell wall synthesis. Thus, we cannot completely exclude the possibility that differences in *N*-glycosylation are possible in the mutant and that such differences modify transcript abundance. A limited number of transcripts were shown recently to be modified in a yeast mutant with decreased *N*-glycosylation capacity (Klebl et al., 2001).

Together, our data strongly indicate that changes in vitamin C contents are the major factor modulating gene expression in *vtc1*. Moreover, the lack of vitamin C may contribute to the dramatic effects on cell wall structure observed in *cyt1* (Lukowitz et al., 2001), because similar effects also are observed in cells deficient only in the last step of vitamin C biosynthesis (Tabata et al., 2001).

Conclusions

This report of widespread modulation of plant gene expression by vitamin C concentration represents an important step toward elucidating the molecular details that explain why this vitamin correlates with growth. Vitamin C contents are lowest in dormant tissues or quiescent cells (Kerk and Feldman, 1995) and are increased markedly in conditions that favor rapid metabolism and growth (e.g., when plants are grown in higher light) (Grace and Logan, 1996; Gillham and Dodge, 1987). One explanation for this finding is that this vitamin is an important buffer against the high oxidative load that accompanies rapid metabolism. Here, we have shown that vitamin C also plays a more active role in which tissue contents affect development via hormonal signaling pathways and modulation of defense networks. Low vitamin C appears to promote slow growth and tip the developmental program in favor of dormancy. Moreover, because low ascorbate makes the mutant much more sensitive to environmental stress, particularly to pollutants such as ozone, different growth conditions could exacerbate these effects. Although we did not conduct our studies in a pure nitrogen/oxygen/carbon dioxide atmosphere, the ambient ozone concentrations (the magnitude of peak concentrations being 20 to 25 parts per billion) in the glasshouses at Rothamsted are well below the levels at which characteristic ozone-induced changes in gene expression can be observed. Moreover, the present study conducted under atmospheric conditions with very low pollution represents a physiological situation that gives a realistic and effective description of ascorbate signaling processes in plants.

The constitutive upregulation of ABA-associated pathways in *vtc1* leaves, independently of effects on photosynthesis, stomatal conductance, or drought stress, explains why the disruption of vitamin C synthesis locks the mutant into a program of slow growth, retarded flowering, and accelerated senescence. The involvement of ABA in the arrest of metabolism and growth suggests that ascorbate sensing might be crucial to plant survival strategies. What is the mechanism? A first possibility is that vitamin C concentration is monitored by specific sensing components that mesh with hormone signaling networks. A second possibility is that tissue concentrations affect the level of enzyme catalysis on hormone contents, given that vitamin C is a cofactor for numerous dioxygenases involved in hormone synthesis (Arrigoni and de Tullio, 2000). Indirect evidence that this may be the case comes from the observed upregulation of transcripts encoding NCED and two 2-oxoglutarate–dependent dehydrogenases.

Low ascorbate induces PR proteins, whereas high ascorbate suppresses their expression. It is remarkable that the defense genes upregulated in *vtc1* do not include any that encode antioxidative enzymes (see supplemental data online). Thus, vitamin C concentration acts as a "crosstalking" signal that coordinates the activity of defense networks complementary to the antioxidant system. An important intermediary in this signaling could be ABA. ABA induces PR1 in a number of species, such as rice (Agrawal et al., 2001), consistent with our data in *vtc1*. However, although wound signals induce

PAL in lettuce, ABA had no effect on PAL activity (Campos-Vargas and Saltveit, 2002). Thus, the regulation of ascorbate-dependent PR genes also may arise by means of hormone signaling.

METHODS

cDNA Preparation

Total RNA was isolated from five independent samples of each of the control and mutant *Arabidopsis thaliana* leaves. The amount and quality of RNA were checked by spectrophotometric measurements and on agarose gels. Ten micrograms of total RNA was used as starting material for the cDNA preparation. First- and second-strand cDNA synthesis were performed using the SuperScript Choice System (Life Technologies, Rockville, MD) according to the manufacturer's instructions except using oligo(dT) primer containing a T7 RNA polymerase promoter site. Labeled complementary RNA (cRNA) was prepared using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY). Biotin-labeled CTP and UTP (Enzo) were used in the reaction together with unlabeled nucleotide triphosphates. After the in vitro transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen, Valencia, CA).

Array Hybridization and Scanning

Arabidopsis microarrays were performed using Affymetrix GeneChip technology (Santa Clara, CA) by the Molecular Diagnostic Laboratory at the Aarhus University Hospital in Denmark. Affymetrix microarray chips contain 8200 genes and 100 EST clusters arranged as 20 probe-paired sequence fragments. Expression analysis techniques were compared using the results from five pairs of array plates. Fifteen micrograms of cRNA was fragmented at 94°C for 35 min in a fragmentation buffer containing 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc. Before hybridization, the fragmented cRNA in 6× SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris, pH 7.6, and 0.005% Triton X-100) (1× SSPE is 0.115 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4) was heated to 95°C for 5 min and subsequently to 40°C for 5 min before loading onto the Affymetrix HuGeneFL probe array cartridge. The probe array was incubated for 16 h at 45°C at constant rotation (60 rpm). The washing and staining procedure was performed in the Affymetrix Fluidics Station.

The probe array was exposed to 10 washes in 6× SSPE-T at 25°C followed by 4 washes in 0.5× SSPE-T at 50°C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate (final concentration, 2 μg/μL; Molecular Probes, Eugene, OR) in 6× SSPE-T for 30 min at 25°C followed by 10 washes in 6× SSPE-T at 25°C. An antibody amplification step was added using normal goat IgG (final concentration, 0.1 mg/mL; Sigma) and anti-streptavidin (goat) biotinylated antibody (final concentration, 3 μg/mL; Vector Laboratories, Burlingame, CA). This step was followed by a staining step with a streptavidin-phycoerythrin conjugate (final concentration, 2 μg/μL; Molecular Probes) in 6× SSPE-T for 30 min at 25°C and 10 washes in 6× SSPE-T at 25°C. The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope with an argon ion laser as the excitation source (GeneArray Scanner G2500A; Hewlett-Packard, Palo Alto, CA).

Bioinformatic Analysis

The readings from the quantitative scanning were analyzed initially using the Affymetrix Gene Expression Analysis Software GeneChip version 3.3. For comparison from array to array, each individual GeneChip was scaled to a global intensity of 150, as described previously (Zhu et al., 1998). As noted elsewhere (Li and Wong, 2001), the currently unavoidable effects of probe-specific bias and variation in microarray analysis can be overcome by appropriate modeling and statistical analyses. Therefore, we reexamined the validity of the primary analysis using dChip software from the Department of Biostatistics at Harvard University (Cambridge, MA; http://biosun1.harvard.edu/~cli/dchip_request.htm) to assemble and reanalyze the array data (Li and Wong, 2001). The readings from the quantitative scanning were analyzed further, and the expression levels were calculated using DNA Chip Analyzer (dChip) enhanced algorithms. The criteria used for the selection of the genes were based on normalized data against the median data image intensities. By default, an array with median overall intensity was chosen as the baseline array against which other arrays were normalized for probe intensity level.

The initial comparison between wild-type and *vtc1* samples was based on the default filtering criterion set by dChip. Four wild-type samples were considered and grouped as "baseline," and four *vtc1* samples were considered and grouped as "experiment." The comparison was performed with a fold change between group means of 1.2 (or 90% lower confidence boundary of fold change) and an absolute difference between group means of 100 at $P < 0.05$. Baseline expression was set to exclude values falling within 20% of median probe intensities. The expression level and pattern were compared against those obtained using the Affymetrix Gene Expression Analysis Software GeneChip version 3.3. Hierarchical clustering was performed using dChip software, and the setting was modified until functionally significant clusters were reached. The functional classification of genes showing modulated expression linked to low vitamin C in *vtc1* was based on the functional organization of the Arabidopsis genome (http://mips.gsf.de/proj/thal/db/index.html).

Photosynthesis and Energy Partitioning

The relationship between total photosynthetic electron flux (J_{II}) and flux linked to ribulose-1,5-bisphosphate carboxylation and oxygenation (J_e) was determined in attached leaves of Arabidopsis illuminated at different irradiances in air. Plants were transferred to the laboratory for simultaneous measurement of steady state CO_2 exchange and chlorophyll fluorescence at different irradiances. These measurements were used to derive two types of photosynthetic activity (Veljovic-Jovanovic et al., 2001) based on established relationships described in detail by von Caemmerer (2000). First, the total rate of electron flow (J_{II}) was calculated from the chlorophyll fluorescence quenching parameter $F'_m - F_v'/F_m'$ (ΦPSII), where F_m' is the relative yield or intensity of chlorophyll fluorescence when all Q_A is reduced in conditions of irradiance and F_v' is the relative change in fluorescence yield or intensity produced in conditions of irradiance relative to F_0, the dark adapted state of fluorescence. Q_A is the primary stable electron acceptor of PSII. ΦPSII is the quantum yield of PSII electron transport. $J_{II} = \Phi PSII \times I \times a \times f$, where I represents irradiance, a represents fractional absorbance of light by the leaf (0.8), and f represents proportion of absorbed energy used by photosystem II (PSII) (0.5). Second, the total number of electrons associated with the processes of carbon fixation and photorespiration (J_e) was calculated from gas-exchange measurements as $J_e = 4(v_c + v_o)$, where v_c and v_o represent rates of ribulose-1,5-bisphosphate carboxylation and oxygenation, respectively. Ribulose-1,5-bisphosphate carboxylase/oxygenase protein and activity were measured on similar rosette leaf material. Total protein corresponds to relative absorption of bands resolved on denaturing gels. Three different parameters were determined for enzyme activity: initial, total, and maximal activities (Parry et al., 1997). Activation state is 100 × (initial activity/total activity).

Abscisic Acid Determinations

The abscisic acid contents of nine independent samples of each of the

control (Columbia [Col0]) and *vtc1* mutant Arabidopsis leaves were measured by radioimmunoassay (Le Page-Degivry et al., 1984).

Reverse Transcriptase-Mediated PCR

Arabidopsis Col0 and *vtc1* plants were grown for 6 to 7 weeks in pots containing a mixture of compost:sand (3:1) in controlled-environment chambers (8-h photoperiod, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance, 60% [v/v] RH, and day/night temperatures of 23 and 18°C). 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, Amp Grade (1 unit/μL; Invitrogen, Carlsbad, CA), and diethyl pyrocarbonate–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 25 mM EDTA followed by incubation at 65°C for 10 min. Synthesis of the first strand of cDNA was performed by adding 1 μL of oligo$(dT)_{12\text{-}18}$ and 0.4 μL of 25 mM deoxynucleotide triphosphate (equimolar solution of dATP, dCTP, dGTP, and dTTP at neutral pH) to 1 μg of total RNA. The mixture then was heated to 65°C during 5 min before being chilled quickly on ice. A brief centrifugation was followed by the addition of 4 μL of 5× first-strand buffer (250 mM Tris-HCl buffer, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$) and 2 μL of 0.1 M DTT and equilibration of the samples at 42°C for 2 min. The transcription initiated from oligo(dT) primers was performed with 200 units of SuperScript II reverse transcriptase (GIBCO) during 50 min at 42°C. The reaction finally was inactivated by heating the mixture at 70°C for 15 min. Samples were stored at −20°C until PCR.

PCR was performed with specific primers for each of the genes analyzed: WRKY binding protein (At4g923180; 5′-AGTGAAGAGTTTGCCGATGG-3′ and 5′-CAGGGAACGAGAAAACGTC-3′), ascorbate oxidase (At4g10500; 5′-TGGCCATCTAGTCCCATCTC-3′ and 5′-GTTGTTGGAGCTTTGAAG-3′), cyclin-dependent kinase (At2g23430; 5′-CTACGGAGCCGGAGAATTG-3′ and 5′-CCATTCGTAACGTCCTTC-3′), NCED (At4g19170; 5′-TTGGTTCTCGAAGGTGGTTC-3′ and 5′-GTCCATCACCAGAAACTTCG-3′), pathogenesis-related protein1 (At2g14610; 5′-AAGCTCAAGATAGCCCACAAG-3′ and 5′-CGTTCACATAATTCCCACGAG-3′), receptor-like kinase a (At4g23260; 5′-CTCTCTCTGTATCAGGTCCACATC-3′ and 5′-GGTTATGTTGGCTATAGGAGACG-3′), receptor-like kinase b (At4g23150; 5′-ACCCTTTTGTCCTCTCTCTCTTC-3′ and 5′-ATAGTGAAGGAGATGCCAACG-3′), receptor-like kinase c (At5g35370; 5′-CAGCCTCTAACCTCAGATTCGTC-3′ and 5′-CCGTTAGATACTCAACAGGGAAG-3′), receptor-like kinase d (At4g04500; 5′-AGTGTCCTCGCCAAAAAGAG-3′ and 5′-GATTGATGACTGAAGGGACCA-3′), and p34 cyclin-dependent kinase 2a (X57839; 5′-TGAAGGAACTTACGGTGTGG-3′ and 5′-TCTCGGAGTCTCCAGGAAAT-3′).

Arabidopsis actin I was used as an internal control 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 mM Tris-HCl, pH 8.4, and 500 mM KCl), 0.4 μL of 25 mM deoxynucleotide triphosphates, 1 μL of each solution of primers (10 mM; forward and reverse), 1.5 μL of 50 mM $MgCl_2$, and 2 units of Taq polymerase. Sterile distilled water was added to a final volume of 50 μL. PCR was performed in a programmable Robocycler (Stratagene, Amsterdam, The Netherlands) at 54°C annealing temperature for all genes. The products of PCR amplification resulted in a single band of the predicted molecular mass. These were analyzed on 1.5% agarose gels against a 1-kb DNA ladder (MBI Fermentas, St. Leon-Rot, Germany) containing the following fragments: 10,000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250 bp. For an accurate comparison of the transcript levels in control and treated samples, PCR cycles were terminated when the products from both the internal control and the gene of interest were detectable and were being amplified within the exponential phase. The exponential phase of amplification occurred at 35 PCR cycles, when the reaction components still were in excess and the PCR products were accumulating at a constant rate. RNA extractions and reverse transcriptase–mediated PCR experiments were performed at least three times.

Ascorbate Feeding Experiment

Arabidopsis Col0 and *vtc1* plants were grown as described above. Leaf discs (1 cm in diameter) were excised and incubated in 10 mM 3-(*N*-morpholino)-propanesulfonic acid buffer, pH 6.0, containing either 0 or 10 mM ascorbate for 16 h in the dark. After the experiment, leaf discs were rinsed, dried, and frozen in liquid nitrogen. Aliquots of 0 and 10 mM ascorbate solutions were taken and used to check the uptake of ascorbate by the leaf discs and the redox state of the solutions before and after the experiment. Ascorbate concentration and redox state were estimated as described by Foyer et al. (1983). At the end of the experiment, *vtc1* treated leaves contained 10-fold higher ascorbate concentrations than *vtc1* control leaves (data not shown). Two leaf RNA samples from *vtc1* control and two from *vtc1* incubated with 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 Cy5, which fluoresces at a green wavelength ($\lambda = 532$ nm), and *vtc1* plus ascorbate was stained with Cy3, which fluoresces at a red wavelength ($\lambda = 635$ nm). The two stained samples were hybridized on the same Arabidopsis cDNA plate. For a detailed description of the microarray experiment, see http://afgc.stanford.edu/afgc_html/AFGCProtocols-Aug2001.pdf. On plate 2, the dye tag was swapped such that *vtc1* responded to red wavelengths and *vtc1* plus ascorbate responded 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 the Axon GenePix system (Axon Instruments, Union City, CA) for each plate of the pair. After visual inspection of the array images and the associated data, the experimental data were normalized using the mechanisms described by Yang et al. (2000) (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 was plotted as an M-versus-A plot, where $M = \log_2(R/G)$, $A = \log_2(\sqrt{(RG)})$, and R and G represent the red and green intensities, respectively, of each spot. This process involves the normalization of the data, taking into account print-tip variations (often referred to as Block variations), using robust Lowess smoothing to remove variation caused by print-tip wear and size, followed by robust scaling of each print tip. The robust scaling suggested by Yang et al. (2000) was a_i^2, where $a_i = MADi/\sqrt[I]{[\prod_{i=1}^{I} MAD_i]}$, $MAD_i = median_j\{|M_{ij} - median_j(M_{ij})|\}$, and MAD is the mean absolute deviation. In this manner, each plate is normalized, and after this normalization process, a normalized M-versus-A plot is produced in which the differentially expressed genes 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 were 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 cutoff of $|meanM| \geq 0.95$ was chosen as an initial boundary level above which reasonable confidence could be given. Follow-up sample reverse transcriptase–mediated PCR confirmed that true expression differences were being observed at these cutoff levels. The spots identified in this analysis are listed in the supplemental data online.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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