Ascorbic Acid Deficiency in *Arabidopsis* Induces Constitutive Priming That is Dependent on Hydrogen Peroxide, Salicylic Acid, and the *NPR1* Gene

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The ascorbic acid (AA)-deficient *Arabidopsis thaliana vtc1-1* mutant exhibits increased resistance to the virulent bacterial pathogen *Pseudomonas syringae*. This response correlates with heightened levels of salicylic acid (SA), which induces antimicrobial pathogenesis-related (PR) proteins. To determine if SA-mediated, enhanced disease resistance is a general phenomenon of AA deficiency, to elucidate the signal that stimulates SA synthesis, and to identify the biosynthetic pathway through which SA accumulates, we studied the four AA-deficient *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* mutants. We also studied double mutants defective in the AA-biosynthetic gene *VTC1* and the SA signaling pathway genes *PAD4*, *EDS5*, and *NPR1*, respectively. All *vtc* mutants were more resistant to *P. syringae* than the wild type. With the exception of *vtc4-1*, this correlated with constitutively upregulated H₂O₂, SA, and messenger RNA levels of *PR* genes. Double mutants exhibited decreased SA levels and enhanced susceptibility to *P. syringae* compared with the wild type, suggesting that *vtc1-1* requires functional *PAD4*, *EDS5*, and *NPR1* for SA biosynthesis and pathogen resistance. We suggest that AA deficiency causes constitutive priming through a buildup of H₂O₂ that stimulates SA accumulation, conferring enhanced disease resistance in *vtc1-1*, *vtc2-1*, and *vtc3-1*, whereas *vtc4-1* might be sensitized to H₂O₂ and SA production after infection.

L-Ascorbic acid (AA, vitamin C) is a multifaceted molecule with diverse physiological functions in plants (Smirnoff 1996) and animals. It is best characterized as an antioxidant that detoxifies reactive oxygen species (ROS), particularly hydrogen peroxide (H₂O₂) (Smirnoff 2000). Under optimal growth conditions, cellular ROS production is low. However, the formation of ROS increases under adverse environmental conditions, including drought, salinity, air pollutants, or pathogen attack (Padh 1990). Because ROS can cause cellular damage, the production and detoxification of ROS is controlled by protective mechanisms (Niyogi 2000; Mittler 2002). However, ROS are also involved in signal transduction pathways controlling pathogen defense responses and programmed cell death (Hammond-Kosack and Jones 1996; Desikan et al. 2001; Neill

et al. 2002). Furthermore, besides being a cosubstrate for AA peroxidase that detoxifies H₂O₂, AA is also a cofactor for 2-oxoacid-dependent dioxygenases that are involved in the biosynthesis of abscisic acid and gibberellins as well as ethylene (Arrigoni and De Tullio 2000, 2002). Research over the past decade has elucidated the plant AA biosynthetic pathway and suggested that AA has crucial functions in addition to its antioxidant properties. These scientific advances would not have been possible without the isolation of AA-deficient *Arabidopsis thaliana* vitamin C-deficient (*vtc*) mutants.

Four *vtc* mutants (*vtc1*, *vtc2*, *vtc3*, and *vtc4*) were originally isolated by Conklin and co-workers (2000). The genes that are defective in these mutants encode enzymes that catalyze steps in the predominant AA biosynthetic pathway, in which glucose is converted to AA via D-mannose and L-galactose (L-Gal) intermediates (Wheeler et al. 1998). The *VTC1* gene encodes a GDP-mannose pyrophosphorylase. The *vtc1-1* mutant contains a point mutation in *VTC1*, resulting in decreased enzyme activity and a 70% lower AA content compared with the wild type (Conklin et al. 1999). A point mutation in the *vtc2-1* mutant causes AA levels that are diminished by 70 to 80% of the wild-type AA content (Conklin et al. 2000; Jander et al. 2002). *VTC2* has GDP-L-Gal phosphorylase (Laing et al. 2007; Linster et al. 2007; Smirnoff et al. 2007) and L-Gal 1-P guanylyl-transferase activity (Laing et al. 2007; Linster et al. 2007). However, *VTC2* GDP-L-Gal phosphorylase activity is predominant and a second enzyme, encoded by *VTC5* with high sequence identity to *VTC2*, also has GDP-L-Gal phosphorylase activity. Both *VTC2* and *VTC5* are required for AA synthesis and seedling viability (Dowdle et al. 2007; Linster et al. 2008). The *VTC3* gene has not yet been identified. *VTC4* encodes an L-Gal-1-phosphate phosphatase (Laing et al. 2004; Conklin et al. 2006). A mutation in this gene causes a 50% lower AA content in *vtc4-1* mutants compared with the wild type.

Physiological characterization of these *vtc* mutants revealed that they are more susceptible to oxidative stress, as expected (Conklin et al. 1997, 2000). However, the *vtc* mutants also exhibit an array of pleiotropic developmental and metabolic defects, including altered flowering and senescence (Pavet et al. 2005; Kotchoni et al. 2009), increased resistance to virulent pathogens (Barth et al. 2004; Pavet et al. 2005), elevated levels of abscisic acid (Pastori et al. 2003; S. O. Kotchoni and C. Barth, *unpublished data*), salicylic acid (SA) (Barth et al. 2004), and a decreased content of gibberellic acid (Kiddle 2004; Foyer et al. 2007).

Increased pathogen resistance was first described in *vtc1-1* and *vtc2-1*, which support less growth of the virulent bacterial

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pathogen *Pseudomonas syringae* pv. *maculicola* ES4326, the cause of leaf spot disease, and the virulent oomycete *Hyaloperonospora parasitica* pv. *Noco*, which causes downy mildew (Barth et al. 2004; Pavet et al. 2005). The enhanced pathogen resistance correlates with higher transcript and protein levels of pathogenesis-related (PR) proteins PR1 and PR5, increased levels of SA, and premature senescence (Barth et al. 2004). It was suggested that AA deficiency promotes expression of genes in the SA defense-signaling pathway and that premature senescence contributes to this enhanced pathogen resistance (Kus et al. 2002). Pavet and associates (2005) investigated whether AA deficiency activates plant innate defense responses via redox mechanisms. The authors reported that the regulatory protein NONEXPRESSOR OF PR1 (NPR1) moves into the nucleus, where it activates the expression of PR genes (Mou et al. 2003) via TGA transcription factors (Despres et al. 2003). The findings by Pavet and associates (2005) led to the conclusion that, through an increased glutathione content and higher redox levels (more glutathione reduced than oxidized), AA deficiency triggers defense responses in the SA signaling pathway, which is independent of H₂O₂. The authors, however, suggested that the defense signaling does not depend on SA.

The SA defense-signaling pathway is activated by avirulent pathogens, causing the hypersensitive response (a form of programmed cell death), and by virulent pathogens, including *P. syringae* and *H. parasitica* (Glazebrook 2001, 2005; Nimchuk et al. 2003). In response to pathogen attack, SA levels rise, resulting in activation of PR genes. SA accumulation requires interaction of PHYTOALEXIN DEFICIENT 4 (PAD4) and ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) (Zhou et al. 1998; Falk et al. 1999; Feys et al. 2001). Both genes encode triacyl-glycerol lipases. However, lipase activity does not appear to be important for the function of either protein (Falk et al. 1999; Jirage et al. 1999; Feys et al. 2001). PAD4 and EDS1 are required for the expression of EDS5, which encodes a MATE family transporter that presumably transports intermediates for SA biosynthesis (Nawrath et al. 2002). Therefore, EDS5 is essential for the accumulation of SA upon pathogen attack (Nawrath and Metraux 1999). The *SID2* gene encodes isochorismate synthase (ICS1), an enzyme in the SA biosynthesis pathway. Mutants with a defect in *SID2* contain strongly reduced levels of SA, suggesting that, in *Arabidopsis*, this hormone is predominantly produced from isochorismate and not phenylalanine, as previously thought (Yalpani et al. 1993; Leon et al. 1995b; Mauch-Mani and Slusarenko 1996; Coquoz et al. 1998; Ribnicky et al. 1998; Wildermuth et al. 2001). Increasing SA levels promote dissociation of NPR1 oligomers into monomers in the cytosol through reduction of disulfide bonds between the NPR1 monomers, which then enter the nucleus. There, the monomers interact with TGA transcription factors to promote expression of PR1 and, presumably, other SA-regulated genes (Zhang et al. 1999; Kinkema et al. 2000; Fan and Dong 2002; Despres et al. 2003; Johnson et al. 2003; Mou et al. 2003; Tada et al. 2008). After localized foliar infections by diverse pathogens, plants often develop whole-plant immunity, known as systemic acquired resistance (SAR) (Durrant and Dong 2004). In this process, which, in addition to pathogen attack, may be induced by treatment with certain natural or synthetic compounds (Conrath et al. 2006; Beckers and Conrath 2007), distal (systemic) leaves become primed to activate a stronger defense response upon secondary infection (Ryals et al. 1996). This type of resistance also depends on the accumulation of SA.

The goal of the current study was to characterize the pathway through which AA deficiency confers enhanced disease resistance to virulent *P. syringae*. Two *vtc* mutants were shown to contain elevated SA levels in the absence of pathogen infec-

tion; therefore, it seems possible that the enhanced disease resistance would be SA-dependent. However, it was previously unknown i) what factors stimulate SA biosynthesis when AA levels are low, ii) through which biosynthetic pathway SA is synthesized and accumulated in *vtc* mutants, and iii) how expression of these traits is influenced by pathogen infection. To address these questions, we measured the SA content in the wild type and four *vtc* mutants and challenged them with *P. syringae*. Furthermore, we generated double mutants between *vtc1-1* and the SA biosynthesis and signaling mutants *pad4-1*, *eds5-1*, and *npr1-1*, respectively, and evaluated bacterial growth and SA content in homozygous single and double mutants. Finally, we determined the H₂O₂ content in *vtc* mutants, the double mutants, and in wild-type and *vtc1-1* plants sprayed with a precursor of AA. Here, we demonstrate that AA deficiency primes plants for enhanced disease resistance that requires H₂O₂, SA, and NPR1.

RESULTS

vtc mutants have constitutively high messenger RNA levels of NPR1 and PR genes that correlate with increased SA levels and enhanced resistance to *P. syringae*.

Rosette leaves of the AA-deficient mutants *vtc1-1* and *vtc2-1* contain approximately 30 and 25% of the wild-type AA content, respectively, whereas the *vtc3-1* and *vtc4-1* mutants have 40 and 50%, respectively (Fig. 1A). As reported previously (Kotchoni et al. 2009), the diminished AA content leads to moderate oxidative stress, which is indicated by higher H₂O₂ levels. The H₂O₂ content in *vtc1-1*, *vtc2-1*, and *vtc3-1* mutants was approximately 60 to 70% higher than in the wild type. In contrast, H₂O₂ levels were the same in the wild type and *vtc4-1* (Fig. 1B).

The *vtc1-1* and *vtc2-1* mutants were reported to have constitutively higher transcript and protein levels of defense genes and increased resistance to virulent pathogens (Barth et al. 2004; Pavet et al. 2005). To test whether this is a general phenomenon of AA deficiency, we measured transcript levels of *NPR1*, *PR1*, *PR2*, and *PR5* in the wild-type and *vtc* mutants. *NPR1* messenger (m)RNA levels were two to three times higher in all *vtc* mutants than in the wild type. Transcript levels of *PR1* were similarly elevated in the *vtc* mutants, except for *vtc4-1*. Compared with the wild type, the *vtc1-1* and *vtc2-1* mutants contained twofold higher transcript levels of *PR2*, whereas *PR2* mRNA levels were unchanged in *vtc3-1* and *vtc4-1*. Finally, *PR5* mRNA levels were more than three times higher in all *vtc* mutants than in the wild type (Fig. 1C).

PR gene expression can be activated through SA-dependent signaling (Shah 2003; Durrant and Dong 2004; Glazebrook 2005). Therefore, we asked whether the higher transcript levels of defense genes correlate with elevated levels of SA and enhanced resistance to virulent *P. syringae*. The *vtc* mutants did not differ in their free SA content. However, the *vtc1-1*, *vtc2-1*, and *vtc3-1* mutants contained approximately 4 and 2.5 times more SA glucoside (SAG), respectively, than the wild type. In contrast, *vtc4-1* had wild-type SA levels (Fig. 1D). The elevated SA content in the mutants correlated with enhanced resistance to *P. syringae* infection in the *vtc* mutants. The *vtc1-1* and *vtc2-1* mutants significantly diminished growth of *P. syringae* 24 h postinoculation, followed by *vtc3-1* that exhibited increased resistance 48 h postinoculation and *vtc4-1*, supporting less growth 72 h postinoculation (Fig. 1E). Note that *vtc4-1* does not contain constitutively elevated SA as found in the other *vtc* mutants. However, it does diminish growth of *P. syringae* at later points in the infection process.

Our data suggest a correlation between the degree of AA deficiency, H₂O₂ and SA accumulation, mRNA levels of defense

genes, and resistance to *P. syringae*. The greater the extent of AA deficiency, the more pronounced are SA-dependent defense signaling and resistance. Because AA levels of 40% or less of the wild type result in a substantial constitutive increase in SA, we asked through which biosynthetic pathway SA is accumulated in the *vtc* mutants.

Double mutants of *vtc1-1* and mutants defective in SA biosynthetic or signaling genes have decreased SA levels and are more susceptible to *P. syringae*.

For pathogen defense in *Arabidopsis*, SA is predominantly synthesized via isochlorismate and not phenylalanine (Wilder-muth et al. 2001). To test whether SA is accumulated in *vtc1-1* through the PAD4-EDS5-SID2-NPR1 biosynthesis and signaling pathway, we generated homozygous double mutants between *vtc1-1*, *pad4-1*, *eds5-1*, and *npr1-1*, respectively. All homozygous double mutants had AA levels similar to those in *vtc1-1* (Fig. 2A). Therefore, we expected that the double mutants also contain higher levels of H₂O₂ that are comparable to *vtc1-1*. This is true for all three double mutants, whereas the

single mutants have H₂O₂ levels similar to the wild type (Fig. 2B). Note, however, that the H₂O₂ content is somewhat lower in the double mutants than in *vtc1-1* single mutants.

If SA is constitutively synthesized via PAD4 and EDS5 in *vtc1-1*, we predicted that *vtc1-1 pad4-1* and *vtc1-1 eds5-1* double mutants would contain low SA levels similar to the SA-biosynthesis *pad4-1* and *eds5-1* single mutants. In contrast, *vtc1-1 npr1-1* double mutants were hypothesized to have increased SA levels, similar to *vtc1-1*. Furthermore, all three double mutants were expected to be more susceptible to *P. syringae* infection due to their inability to either accumulate SA (*vtc1-1 pad4-1* and *vtc1-1 eds5-1*) or mediate PR gene induction (*vtc1-1 npr1-1*). As expected, *vtc1-1 pad4-1* and *vtc1-1 eds5-1* double mutants were SA-deficient, containing free SA (Fig. 2C) and SAG (Fig. 2D) levels similar to the *pad4-1* and *eds5-1* single mutants. Unexpectedly, the *vtc1-1 npr1-1* double mutant had an SAG content that was approximately twice as high as that of the wild type and *npr1-1* single mutants (Fig. 2D), whereas the double mutant did not differ from the wild type in respect to the amount of free SA (Fig. 2C). However,

Fig. 1. Physiological characterization of 3-week-old ascorbic acid (AA)-deficient vitamin C-deficient (*vtc*) mutants. **A**, Total AA and **B**, hydrogen peroxide (H₂O₂) content based on gram fresh weight (FW) in whole rosettes of Columbia-0 wild-type (Col WT) and *vtc* mutant plants. **C**, Transcript levels of *NPR1*, *PR1*, *PR2*, and *PR5* normalized to *ACTIN*. **D**, Free salicylic acid (SA) and SA-glucoside (SAG, the conjugated form) in whole rosettes of wild-type and *vtc* mutant plants. **E**, Bacterial growth of virulent *Pseudomonas syringae* (expressed in CFU per square centimeter of leaf area) in the wild type and *vtc* mutants. Three-week-old plants were syringe-infiltrated with a bacterial suspension of 10⁵ CFU ml⁻¹ and leaf discs were collected at the indicated time points. Asterisks indicate significant differences between the wild type and mutants: *, **, and *** = *P* < 0.05, 0.01, and 0.001, respectively, Student's *t* test.

the SAG content, although significantly higher than in the wild type, was more than two times lower in *vtc1-1 npr1-1* mutants compared with *vtc1-1* single mutants (Fig. 2D).

As hypothesized, all three double mutants supported more growth of *P. syringae* than the wild type and *vtc1-1*, the latter being the most resistant. The double mutants were susceptible to *P. syringae* infection similarly to the single mutants (Fig. 3A). We also investigated the accumulation of H₂O₂ in the various genotypes during the infection process in order to test the effect of the *vtc1-1* mutation in the double mutants. Knowledge about the H₂O₂ content is also important, because SA is known to not only potentiate H₂O₂ accumulation (Shirasu et al. 1997) but also to act downstream of H₂O₂ (Leon et al. 1995a; Neuenschwander et al. 1995). As an example, the H₂O₂ content in the wild type, *vtc1-1* and *npr1-1* single mutants, and *vtc1-1 npr1-1* double mutants is illustrated in Figure 3B. All genotypes responded to *P. syringae* infection with an increase in H₂O₂ up to 48 h after

inoculation. This increase was most pronounced in the wild type, which had even higher H₂O₂ levels 72 h postinfection, whereas no further increase was observed in *vtc1-1*. H₂O₂ levels decreased in both *npr1-1* and *vtc1-1 npr1-1* mutants 72 h after inoculation (Fig. 3B) when peak bacterial density was reached in these mutants (Fig. 3A), which may be a result of demolished leaf integrity due to *P. syringae* infection.

Compared with mock-inoculated controls (Fig. 3C and D) and despite the SA biosynthesis or signaling defect, single and double mutants were able to induce free SA (Fig. 3E) and SAG (Fig. 3F), to some extent, 72 h postinoculation. The difference in SAG content between the wild type and *vtc1-1* was less pronounced 72 h after infection, because the wild type induces SA accumulation. Large differences were observed in the SAG content in *npr1-1* single and *vtc1-1 npr1-1* double mutants, which contained approximately five times higher SAG levels than the wild type (Fig. 3F).

Fig. 2. Ascorbic acid (AA) and hydrogen peroxide (H₂O₂) content in the wild type and double mutants with defects in AA and salicylic acid (SA) biosynthesis or signaling. **A**, Total AA; **B**, H₂O₂ content; **C**, free SA; and **D**, SA-glucoside (SAG) content based on gram fresh weight (FW) in whole rosettes of 3-week old Columbia-0 wild type (Col WT), vitamin C-deficient (*vtc1-1*), *pad4-1*, *eds5-1*, and *npr1-1* single mutants and *vtc1-1 pad4-1*, *vtc1-1 eds5-1*, and *vtc1-1 npr1-1* double mutants under normal growth conditions. Asterisks indicate significant differences between the wild type and mutants: *, **, and *** = $P < 0.05$, 0.01, and 0.001, respectively, Student's *t* test.

Fig. 3. Bacterial growth, hydrogen peroxide (H₂O₂), and salicylic acid (SA) content in Columbia-0 wild-type (Col WT), vitamin C-deficient (*vtc1-1*), *pad4-1*, *eds5-1*, and *npr1-1* single mutants and *vtc1-1 pad4-1*, *vtc1-1 eds5-1*, and *vtc1-1 npr1-1* double mutant plants. **A**, Bacterial growth curve of *Pseudomonas syringae*. Three-week-old plants were inoculated by dipping using a bacterial suspension of 10⁷ CFU ml⁻¹. Leaf discs were collected at the indicated time points. **B**, H₂O₂ content based on gram fresh weight (FW) throughout the infection process. **C**, Free SA and **D**, SA glucoside (SAG) content based on gram FW in wild-type and mutant plants in mock-infected plants 72 h after the start of infection. **E**, SA and **F**, SAG content based on gram FW in wild-type and mutant plants 72 h after *P. syringae* inoculation. Asterisks indicate significant differences between the wild type and mutants: *, **, and *** = *P* < 0.05, 0.01, and 0.001, respectively, Student's *t* test.

In summary, these data suggest that the activity of PAD4, EDS5, and NPR1 is required for the accumulation of SA and enhanced resistance against *P. syringae* in *vtc1-1*. To further investigate the intriguing role of NPR1 in the accumulation of H₂O₂ and SA, we performed a developmental analysis of the H₂O₂ and SA content in *vtc1-1 npr1-1* double mutants. Furthermore, to establish whether H₂O₂ functions upstream of SA and, thus, promotes the constitutive accumulation of SA in *vtc1-1*, we tested whether the accumulation of H₂O₂ and SA is attenuated when AA levels are increased artificially or in vivo in a *vtc1-1*-suppressor mutant.

The presence of the *npr1-1* mutation in *vtc1-1 npr1-1* double mutants suppresses H₂O₂ and SA accumulation in the absence of pathogen infection.

To investigate whether SA accumulation is suppressed in *vtc1-1 npr1-1* because of suppressed H₂O₂ levels or because of alterations throughout development, we analyzed the H₂O₂ and SA contents in the wild type, *vtc1-1*, *npr1-1*, and *vtc1-1 npr1-1* mutants when plants were 2, 3, and 4 weeks old.

As expected and as reported previously (Kotchoni et al. 2009), H₂O₂ levels increased throughout development in all genotypes. Although H₂O₂ levels were slightly but significantly higher in *vtc1-1 npr1-1* double mutants compared with the wild type in 2- and 3-week-old and unchanged in 4-week-old plants, H₂O₂ content was significantly lower in the double mutant when compared with *vtc1-1* when plants were 3 and 4 weeks old (Fig. 4A). Although the free SA content was largely similar among all genotypes at the three developmental stages tested (Fig. 4B), the SAG content was approximately three to six times higher in *vtc1-1* mutants than in the wild type at all developmental stages (Fig. 4C). Generally, SA content decreased in all genotypes when plants were 4 weeks old. The SAG content was largely unchanged in the wild type and *vtc1-1 npr1-1* mutants but decreased in *vtc1-1* (Fig. 4C). Thus, the decline in SAG content in *vtc1-1 npr1-1* compared with *vtc1-1* single mutants was most pronounced in 2-week-old plants. Note, however, that this change did not correlate with an altered H₂O₂ content at this developmental stage.

Higher AA pool size decreases H₂O₂ and SA content.

AA content was artificially increased by feeding the AA precursor L-Gal to wild-type and *vtc1-1* mutant plants. If H₂O₂ induces SA accumulation, an increase in AA pool size should result in a decrease in H₂O₂ and SA levels.

L-Gal spraying doubled the total AA content in the wild type and complemented the AA-deficiency phenotype in *vtc1-1* compared with the water-sprayed control plants (Fig. 5A). The H₂O₂ content was similar in both genotypes after L-Gal treatment and was significantly lower in both the wild type and *vtc1-1* compared with plants sprayed with water (Fig. 5B). Although no significant change after L-Gal treatment was observed in the SA content in either genotype, the decreased H₂O₂ content in *vtc1-1* correlated with a decrease in SAG content. However, a similar trend was not observed in the wild type (Fig. 5C).

Further support for a role of H₂O₂ in affecting SA biosynthesis or accumulation comes from the analysis of the recently identified partial suppressor mutant *vtc1-1 fca-1* (Kotchoni et al. 2009). In addition to the *vtc1-1* mutation, the *vtc1-1 fca-1* double mutant contains a mutation in the autonomous flowering pathway gene *FCA-1*. The double mutant has approximately 75% of the Col/Ler wild-type AA content whereas, compared with *vtc1-1*, the AA content is twice as high (Fig. 6A). Although the mechanism leading to the partially rescued AA content is presently unknown, we expected that H₂O₂ levels would be lower in *vtc1-1 fca-1* double mutants. The H₂O₂ content de-

creased to almost Col/Ler wild-type levels in the double mutant (Fig. 6B). Thus, if H₂O₂ acts upstream of SA, we predicted that the *vtc1-1 fca-1* double suppressor mutant contains wild-type levels of SA. This is indeed the case (Fig. 6C).

In sum, the results presented in Figures 5 and 6 suggest that low levels of AA contribute to the formation of H₂O₂, resulting in the promotion of SA biosynthesis or accumulation in *vtc1-1*.

Fig. 4. Hydrogen peroxide (H₂O₂) and salicylic acid (SA) content throughout development in whole rosettes of the Columbia-0 wild type (Col WT), vitamin C-deficient (*vtc1-1*) and *npr1-1* single mutants, and *vtc1-1 npr1-1* double mutants. **A**, H₂O₂ content based on gram fresh weight (FW). **B**, Free SA and **C**, SA glucoside (SAG) per gram FW. Asterisks indicate significant differences between the wild type and mutants: *, **, and *** = *P* < 0.05, 0.01, and 0.001, respectively, Student's *t* test.

DISCUSSION

AA deficiency induces priming, allowing for a more rapid induction of SA-mediated defense signaling.

Evidence reported here suggests that AA deficiency causes constitutive priming. Low levels of AA lead to increased SA levels, higher mRNA levels of *PR* genes, and enhanced resistance to *P. syringae*. The *Arabidopsis vtc* mutants resemble an intermediate phenotype between the constitutively primed *edr1*, *mpk4*, *cpr1*, and *cpr5* mutants. Although *PR1* transcripts were hardly detected in *edr1* (Frye and Innes 1998; Frye et al. 2001; van Hulten et al. 2006) in the absence of pathogens, *mpk4*, *cpr1*, and *cpr5* exhibited constitutively high mRNA levels of *PR1* (Petersen et al. 2000; Wildermuth et al. 2001; van Hulten et al. 2006). Elevated *PR1* transcripts were observed in *vtc* mutants (except for *vtc4-1*), although mRNA levels were not as strongly elevated as in *mpk4* and *cpr1* mutants (Fig. 1C). However, *PR1* and *PR5* protein levels were not constitutively higher in *vtc1-1* (Barth et al. 2004). SA levels are much higher in *mpk4* and *cpr1* than in *vtc* mutants (Fig. 1D) and mRNA levels of *SID2* and *PAL1* are constitutively higher in *cpr1* mutants (Wildermuth et al. 2001; Kohler et al. 2002), which was not observed in *edr1-1* (Beckers et al. 2009) and *vtc1-1* mutants in the absence of priming agents or pathogens (data not shown). Yet, upon *P. syringae* infection, *vtc* mutants exhibited varying degrees of disease resistance (Fig. 1E).

In addition to SA, the key SA-signaling regulator *NPR1* (Ahn et al. 2007; Beckers et al. 2009) and mitogen-activated protein kinase genes, including *MPK3* and the functionally redundant *MPK6* (Beckers et al. 2009), are required for priming. Our analysis of mutants with defects in the AA biosynthetic gene *VTC1* and the SA biosynthetic genes *PAD4* and *EDS5* as well as the SA signaling gene *NPR1* demonstrated that the constitutively elevated SA content in the *vtc1-1* mutant requires *PAD4* and *EDS5* and, to some extent, *NPR1* (Figs. 2D and 4C). Accumulation of SA in the *vtc1-1*, *vtc2-1*, and *vtc3-1* mutants is determined by an increase in the conjugated form of SA, SAG (Fig. 1D). It is possible that SA glycosyl transferase, an enzyme that converts free SA to SAG, is activated in these mutants in order to reduce the amount of active SA (Lee et al.

1995). We hypothesized that SA is synthesized via the isochorismate synthase (*ICS1* or *SID2*) and not the phenylalanine pathway, because it is known that the former is induced upon local and systemic pathogen infection (Wildermuth et al. 2001; Attaran et al. 2009). Although we did not generate double mutants between *vtc1-1* and *sid2* mutants, our data unequivocally show that constitutive accumulation of SA in *vtc1-1* occurs through the isochorismate pathway. This is supported by the fact that the *PAD4* and *EDS5* genes are upstream of *SID2* (Nawrath et al. 2002), whereas *NPR1-1* acts downstream of *SID2* (Shah 2003; Glazebrook 2005). Hence, priming in *vtc1-1*, and most likely in *vtc2-1* and *vtc3-1*, is dependent on SA biosynthesis mediated by *PAD4*, *EDS5*, and *SID2*, as well as *NPR1*, because *pad4-1*, *eds5-1*, and *npr1-1* mutations suppress SA accumulation and resistance in *vtc1-1* (Fig. 3A).

Assessment of transcript levels of *PAL1*, *SID2*, *MKK2*, *MPK3*, *MPK4*, *MPK6*, and *EDR1* genes that have been suggested to play a role in priming and whose expression is altered in constitutively primed plants did not reveal a significant upregulation of these genes in *vtc1-1* in the absence of pathogen challenge (M. Mukherjee, T. S. Bedick, N. J. Ahmed, C. Barth, unpublished results). Therefore, we suggest that priming in *vtc* mutants is mediated through a somewhat different signaling pathway. The enhanced disease resistance in *vtc* mutants may reflect an overlap between SA-induced defense responses and the genetic control of senescence. A similar mechanism has been suggested for the *edr1-1* mutant (Frye et al. 2001; Tang and Innes 2002). This is supported by the following facts: i) *vtc1-1* enters senescence prematurely (Barth et al. 2004); ii) several defense genes are induced during leaf senescence, including *PR1* (Hanfrey et al. 1996; Morris et al. 2000); and iii) the senescence process is partially regulated by SA, because *Arabidopsis* mutants defective in SA signaling, such as *npr1-1*, *pad4*, and NahG transgenic plants, exhibit delayed yellowing and reduced necrosis (Morris et al. 2000).

Our data suggest that *vtc1-1*, *vtc2-1*, and *vtc3-1* can induce defense against *P. syringae* more rapidly through SA-dependent upregulation of *PR* genes. This, however, may not be the case in *vtc4-1*, which responds more subtly to *P. syringae* infection, not allowing us a simple conclusion as to what defense

Fig. 5. Effect of L-galactose (L-Gal) on ascorbic acid (AA), hydrogen peroxide (H_2O_2), and salicylic acid (SA) content in 3-week-old plants of the Columbia-0 wild type (Col WT) and vitamin C-deficient (*vtc1-1*) mutants. **A**, Total AA content based on gram fresh weight (FW) in L-Gal- and water-treated plants. **B**, H_2O_2 content. **C**, Free SA and SA glucoside (SAG) content in the wild type and *vtc1-1* mutants treated with water or L-Gal. Asterisks indicate significant differences between the wild type and *vtc1-1* or between treatments: *, **, and *** = $P < 0.05$, 0.01, and 0.001, respectively, Student's *t* test.

mechanisms are activated in this mutant (Fig. 1E). The *vtc4-1* mutant, in addition to *vtc1-1* and *vtc2-1*, has significantly increased ionically bound peroxidase activity, suggesting that these mutants have higher cell wall peroxidase activity (Colville and Smirnoff 2008). Cell wall peroxidase activity, which determines cell wall structure and mechanical properties through peroxidative cross-linking of cell wall proteins and polysaccharides (Brady and Fry 1997), may contribute to cell wall strengthening in response to pathogens (Lamb and Dixon 1997). Cell wall peroxidase can also generate H_2O_2 , as was demonstrated in antisense plants with downregulated peroxidase, resulting in decreased apoplastic H_2O_2 and increased sensitivity to virulent and avirulent pathogens (Bindschedler et al. 2006). Therefore, altered cell wall composition and the induction of H_2O_2 after pathogen infection may contribute to the induction of defense responses in *vtc4-1*. Furthermore, it was demonstrated recently that *VTC4* is a bifunctional enzyme that not only affects AA but also myoinositol biosynthesis (Torabinejad et al. 2009). Myoinositol is incorporated into many crucial cellular compounds, such as phosphatidylinositol phosphate and myoinositol phosphate, that are involved in signal transduction (Boss et al. 2006). Therefore, it is possible that other signals are important for priming in *vtc4-1*. This is supported by reports suggesting that establishment of SAR does not always correlate with an accumulation of SA and the mobile signal methyl salicylate (Cameron et al. 1999; Attaran et al. 2009). Furthermore, a study by Ahn and associates (2007) indicated that strong and long-lasting *PR1* transcription by treatment with vitamin B1, which induces priming, is not crucial for the induction of SAR. Finally, pathogen defense in the absence of *PR1* expression was reported in wheat (Gorlach et al. 1996) and barley (Jarosch et al. 2003) treated with the priming-inducing agent BTH, an SA functional analog, as well as in tobacco and rice treated with brassinolide (Nakashita et al. 2003).

AA deficiency-induced priming requires NPR1 and H_2O_2 in *vtc1-1*.

As indicated above, NPR1 is required for priming in *vtc1-1*. This is not surprising, because the *npr1* mutant is priming-deficient (Kohler et al. 2002; Beckers et al. 2009). However, our data suggest a more complex function of NPR1 in regulating enhanced disease resistance in *vtc* mutants.

Accumulation of SA is higher in pathogen-infected *npr1* mutants than in pathogen-inoculated wild-type plants (Fig. 3F) (Delaney et al. 1995; Shah et al. 1997; Attaran et al. 2009). This suggests that NPR1 plays a role in responding to elevated SA levels and controlling SA content. Our results support this function. Upon *P. syringae* infection, *vtc1-1 npr1-1* double mutants contain substantially higher SAG levels than the wild-type and *vtc1-1* single mutants (Fig. 3F). Because of the genetic defect in NPR1, the protein does not function to induce defense genes in *npr1-1* single and *vtc1-1 npr1-1* double mutants. Thus, despite the accumulation of SA upon pathogen challenge, disease cannot be prevented in these mutants. This further supports the essential function of NPR1 in enhanced defense responses in *vtc1-1* and is consistent with what has been reported previously (Pavet et al. 2005). A more difficult question to answer is why the SAG content is suppressed in *vtc1-1 npr1-1* double mutants in the absence of *P. syringae* (Figs. 2D, 3D, and 4C).

It is known that defense activators and subsequent pathogen infection trigger the formation of ROS, acting as signaling molecules stimulating SAR (Tenhaken et al. 1995; Wojtaszek 1997; Alvarez et al. 1998). Specifically, H_2O_2 induces defense activation and enhanced pathogen tolerance in tobacco (Chamnongpol et al. 1998) and is required for priming (Ahn et al. 2007). The priming-deficient *npr1* mutant fails to accumulate H_2O_2 after

Fig. 6. Ascorbic acid (AA), hydrogen peroxide (H_2O_2), and salicylic acid (SA) content in the vitamin C-deficient (*vtc1-1 fca-1*) partial suppressor mutant. **A**, Total AA content based on gram fresh weight (FW) in Columbia-0 wild type (Col WT), *vtc1-1* (Col-0 background), Landsberg *erecta* wild type (Ler WT), the autonomous flowering pathway mutant *fca-1* (Ler background), a Col/Ler wild-type cross, and *vtc1-1 fca-1* double mutants (Col/Ler background). **B**, H_2O_2 content in the wild types and single and double mutants. **C**, Free SA and SA glucoside (SAG) content in the wild types and single and double mutants. Asterisks indicate significant differences between the wild type and mutant: ** and *** = $P < 0.01$ and 0.001 , respectively, Student's *t* test.

priming with thiamine (vitamin B1) and challenge with *P. syringae* DC 3000 (Ahn et al. 2007). Furthermore, H₂O₂ has been shown to induce SA biosynthesis in tobacco (Leon et al. 1995a; Neuenschwander et al. 1995). Our experiments revealed that SAG but not free SA levels are repressed in *vtc1-1 npr1-1* double mutants, particularly at early developmental stages (Figs. 2D and 4C). However, our data do not support a relationship between H₂O₂, SA content, and the function of NPR1. Although the *npr1-1* mutation suppresses H₂O₂ accumulation in *vtc1-1 npr1-1* mutants to some extent (Figs. 2B and 4A), H₂O₂ content does not correlate with the suppression in SAG content in uninfected *vtc1-1 npr1-1* double mutants (compare Fig. 4A and C). This suggests that alterations in the SAG content in *vtc1-1 npr1-1* double mutants are independent of H₂O₂. This is further supported by the fact that *npr1-1* and *vtc1-1 npr1-1* mutants have low levels of H₂O₂ 72 h post*P. syringae* infection, whereas SA content is greatly elevated (compare Fig. 3B and F). The decrease in H₂O₂ content may simply be explained by a loss of leaf integrity in *npr1-1* and *vtc1-1 npr1-1*. However, one might then also expect to see a decrease in SA content at this point in the infection process. This, however, is not the case. It is not clear whether antioxidant enzymes are activated in *npr1-1* and *vtc1-1 npr1-1*, thus contributing to the decrease in H₂O₂ content.

We can only speculate as to how NPR1 functions in the regulation of SA content in *vtc1-1 npr1-1* double mutants. Clearly, NPR1 serves as a negative regulator of SA biosynthesis when plants are challenged with *P. syringae* (Delaney et al. 1995; Shah et al. 1997; Attaran et al. 2009). However, NPR1 is required for the constitutive accumulation of SA in uninfected *vtc1-1* (Figs. 2D and 4C). It has been suggested previously that SA and NPR1 play broader roles in cell fate control and that NPR1 promotes cell division or represses endoreduplication during leaf development (Vanacker et al. 2001). This seemingly dual role of NPR1 may be explained by recent findings that suggest a role of chromatin remodeling in priming of SA-responsive loci in SAR (van den Burg and Takken 2009). These studies suggest that prolonged SA accumulation upon pathogen challenge leads to NPR1-dependent repression of SA synthesis in a histone deacetylase 19 (HDA19)-dependent manner. This would explain why *npr1-1* mutants accumulate SA upon pathogen inoculation (Fig. 3F). Yet, it does not explain why SA levels are suppressed in *vtc1-1* by *npr1-1* in the absence of *P. syringae*. A correlation between SA, glutathione (GSH), and H₂O₂ contents in plants has been reported. Constitutive pathogen resistance in the *cpr1* and *cpr5* mutants correlated with higher SA, GSH, and H₂O₂ content (Mateo et al. 2006). Mateo and co-workers suggested that high SA is associated with high GSH levels. Therefore, one would expect a substantially higher GSH content in *vtc1-1*, which contains approximately two times more free SA and eight times more SAG than the wild type when plants are only 2 weeks old (Fig. 4B and C). However, Conklin and co-workers (1996) did not find that AA deficiency in *vtc1-1* affects accumulation of reduced or oxidized glutathione. Likewise, Pavet and co-authors (2005) only reported an approximately 1.4 times higher GSH content in *vtc1-1* and no change in GSH and a rather lower GSH redox status in *vtc2-1* when compared with the wild type when plants were 2 weeks old. Similar results were reported by Colville and Smirnov (2008). Consequently, the GSH pool is only affected to a small degree in the *vtc* mutants. Therefore, we suggest that the high SA content and NPR1 signaling are independent of GSH in *vtc1-1*. Although a role of GSH in promoting NPR1 monomerization has been reported previously (Mou et al. 2003; Pavet et al. 2005), it is now known that GSH is not the direct reducing agent of NPR1 in vivo and that thioredoxins catalyze NPR1 oligomer-to-monomer formation (Tada et al. 2008).

Although the function of NPR1 in regulating SA content in *vtc* mutants requires further investigation, we established a role of H₂O₂ in the induction of SA when AA is low (Figs. 5 and 6). Our L-Gal spray experiments and investigation of the *vtc1-1 fca-1* suppressor mutant support the fact that H₂O₂ acts upstream of SA and contributes to SA induction in *vtc1-1*. However, there may be additional factors that stimulate SA biosynthesis. Our data are in contrast to those of Pastori and associates (2003), who previously suggested that the induction of PR proteins and disease resistance in *vtc1-1* is independent of H₂O₂ and SA, because H₂O₂ content was found to be similar to the wild type (Veljovic-Jovanovic et al. 2001) and *PAL* mRNA levels were not upregulated. Perhaps the H₂O₂ assay used here is more sensitive than the method used by Veljovic-Jovanovic and associates (2001) and Pastori and associates (2003). Also, Pastori and co-workers did not measure the SA content.

In conclusion, when foliar AA levels are 40% or less of the wild type, formation of H₂O₂ is induced, resulting in isochorismate synthase-dependent accumulation of SA that also requires the function of NPR1. This may allow AA-deficient plants to adjust redox homeostasis, thereby allowing plants to optimize growth and development in response to environmental changes, including virulent pathogens.

MATERIALS AND METHODS

Plant material and growth conditions.

A. thaliana wild-type, ecotype Columbia (Col-0), *vtc*, and *npr1-1* mutant seed were provided by P. Conklin (SUNY Cortland). Mutant seed of *pad4-1* and *eds5-1* were provided by G. Jander (Boyce Thompson Institute for Plant Research). These mutants are in the Col-0 background. In addition, the wild-type Landsberg *erecta* (Ler-0, CS20) and *fca-1* (CS167 in the Ler-0 background) were obtained from the Arabidopsis Biological Resource Center. Double mutant plants of *vtc1-1 pad4-1*, *vtc1-1 eds5-1*, *vtc1-1 npr1-1*, and *vtc1-1 fca-1* were generated in the Barth laboratory as described below. Wild-type and mutant plants were grown in a growth chamber (Percival, Perry, IA, U.S.A.). Plants were grown in Metromix 360 soil (BFG Supplies, Burton, OH, U.S.A.). Seed of wild types and mutants were planted randomly in 32-insert flats. Plants were grown under long days (16 h of light and 8 h of darkness) at a light intensity of 160 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (fluorescent bulbs) and a temperature of 23°C during the day and at night. Generally, whole rosettes of 3-week-old plants were harvested unless indicated otherwise. Leaf tissue was immediately frozen in liquid nitrogen and kept at -80°C for later analysis.

Generation and identification of double mutants.

The *vtc1-1* mutant was crossed with *pad4-1*, *eds5-1*, *npr1-1*, and *fca-1*, respectively, as described previously (Kotchoni et al. 2009). Per plant, two polymerase chain reaction (PCR) reactions were carried out to identify homozygous double mutants using the primers listed in Supplementary Table S1. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.) and sequenced at Macrogen, Inc. (Seoul, Korea). F₃ and F₄ plants of homozygous double mutants were used for experimental analyses.

Bacterial infection

with virulent *P. syringae* pv. *maculicola* ES4326.

The virulent strain of *P. syringae* pv. *maculicola* ES4326 was provided by G. Martin (Boyce Thompson Institute for Plant Research). Inoculation of plants was performed using the syringe infiltration (Barth et al. 2004) or the dipping method (Katagiri et al. 2002) using an inoculation titer of 10⁵ and 10⁷ CFU ml⁻¹, respectively. Mock controls were either infiltrated

with 10 mM MgCl₂ or dipped in water containing 0.04% of the surfactant Silwet L-77 (Lehle Seeds, Round Rock, TX, U.S.A.).

Exogenous application of L-Gal.

Wild-type and *vtc1-1* mutant plants were grown as described above. Seven days after germination, plants were sprayed every day with either water or 10 mM L-Gal dissolved in water. The galactose solution (10 ml) was sprayed onto one flat containing 32 plants, and each plant received two sprays (approximately 150 µl). When plants were 3 weeks old, rosette leaves were harvested for determination of AA, H₂O₂, and SA content.

AA content assay.

The amount of total and reduced AA was determined in whole rosettes of 3-week-old plants (fresh weight was approximately 100 to 200 mg) following the iron reduction assay (Kampfenkel et al. 1995; Dowdle et al. 2007). To account for absorbance caused by the presence of anthocyanins, an additional control not containing dipyriddy was included for samples and standards. Absorbance values of the samples were corrected by subtracting the absorbance value of the blank and the dipyriddy controls.

SA content assay.

Total and free SA were measured from whole rosettes (200 to 300 mg fresh weight) essentially as described previously (Bowling et al. 1994). Briefly, after extracting the tissue in 2.5 ml of 90% methanol, 50 µl of 0.1 mM O-anisic acid (Sigma-Aldrich, St. Louis) were added as an internal standard. Samples were shaken for 20 min at room temperature and centrifuged at 2,800 × g for 15 min. The supernatant was transferred to a new glass tube and the previous steps were repeated after resuspending the pellet in 100% methanol. The supernatants were combined, mixed, split into two fractions, and dried under vacuum in a glass desiccator for 2 days. To purify SA, 400 µl of 100 mM sodium acetate at pH 5.5 was added to samples for measuring free SA, and 40 U of β-glucosidase suspended in sodium acetate were added to samples for measuring total SA. Samples were incubated at 37°C for 1.5 h. An equal volume of 50% trichloroacetic acid was added to the samples followed by centrifugation at 2,800 × g for 15 min at room temperature. The supernatants were then partitioned twice with 1.25 ml of a mixture (100:99:1) of ethylacetate/cyclopentane/2-propanol. The organic phase was collected and dried under vacuum for 2 to 3 days. The dried samples were stored at -20°C (no more than 1 week) until samples were analyzed by high-performance liquid chromatography (HPLC). The pellet was suspended in 55% methanol and the samples were passed through 0.2-µm nylon spin columns (Fisher, Pittsburgh) by centrifugation at 14,000 × g for 2 min. Samples were then analyzed on an HP1100 HPLC System (Agilent, Santa Clara, CA, U.S.A.) equipped with a 4.6-by-150-mm Zorbax Eclipse Column and fluorescence detector (excitation at 301 nm and emission at 412 and 365 nm for SA and O-anisic acid, respectively). Solvent flow was 1 ml min⁻¹. Mobile phases were i) 100% methanol and ii) 0.5% acetic acid. The solvent program consisted of 0 to 5 min at i) 30% and ii) 70%, followed by a linear increase to i) 40% and ii) 60% at 7.5 min and i) 60% and ii) 40% at 15 min, returning to i) 30% and ii) 70% at 18 min.

H₂O₂ content assay.

H₂O₂ production in whole rosettes of the wild type and mutants was measured as previously described (Shin and Schachtman 2004), using the Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA, U.S.A.).

Extraction of RNA, cDNA synthesis, and gene expression analysis.

Isolation of total RNA, cDNA synthesis, determination of transcript levels using semiquantitative PCR, and quantification of PCR products of defense-related genes was performed according to Kotchoni and associates (2009). Gene-specific primers are listed in Supplementary Table S2. A cDNA fragment generated from *ACTIN* or *TUBULIN* served as an internal control.

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