Alterations in the Endogenous Ascorbic Acid Content Affect Flowering Time in Arabidopsis[1[W][OA]]

Simeon O. Kotchoni[2], Katherine E. Larrimore[3], Madhumati Mukherjee[3], Chase F. Kempinski, and Carina Barth*

Department of Biology, West Virginia University, Morgantown, West Virginia 26506

Ascorbic acid (AA) protects plants against abiotic stress. Previous studies suggested that this antioxidant is also involved in the control of flowering. To decipher how AA influences flowering time, we studied the four AA-deficient Arabidopsis (*Arabidopsis thaliana*) mutants *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* when grown under short and long days. These mutants flowered and senesced before the wild type irrespective of the photoperiod, a response that cannot simply be attributed to slightly elevated oxidative stress in the mutants. Transcript profiling of various flowering pathway genes revealed a correlation of altered mRNA levels and flowering time. For example, circadian clock and photoperiodic pathway genes were significantly higher in the *vtc* mutants than in the wild type under both short and long days, a result that is consistent with the early-flowering phenotype of the mutants. In contrast, when the AA content was artificially increased, flowering was delayed, which correlated with lower mRNA levels of circadian clock and photoperiodic pathway genes compared with plants treated with water. Similar observations were made for the autonomous pathway. Genetic analyses demonstrated that various photoperiodic and autonomous pathway mutants are epistatic to the *vtc1-1* mutant. In conclusion, our transcript and genetic analyses suggest that AA acts upstream of the photoperiodic and autonomous pathways.

In higher plants, the timing of the transition from the vegetative to the reproductive phase is essential to ensure reproductive success. Flowering time is controlled by external and internal factors that are integrated in a complex gene regulatory network that ensures the expression of flowering genes, resulting in flower formation (Jack, 2004; Corbesier and Coupland, 2005). Environmental factors that regulate flowering include daylength, light, and temperature. The plant hormone GA is an important internal factor that controls flowering. Therefore, we differentiate four flowering pathways in the facultative long-day plant Arabidopsis (*Arabidopsis thaliana*): the photoperiodic, vernalization, autonomous, and GA pathways.

One of the most important environmental factors that affect floral transition is the change in daylength (photoperiod). A role of photoperiod was originally proposed by Tournois and Klebs (Tournois, 1912; Klebs, 1913). In the 1920s, Garner and Allard (1920, 1923) were the first who discovered that flowering and other developmental responses could be controlled by exposure to short day (SD) or long day (LD) depending on the plant species. They introduced the terms "photoperiod," which defines the recurring duration of daily light and dark periods, and "photoperiodism," which defines the responses to photoperiod. Numerous studies have been devoted to elucidating the molecular mechanisms of the photoperiodic flowering pathway (for comprehensive reviews, see Imaizumi and Kay, 2006; Kobayashi and Weigel, 2007). This pathway, which consists of a circadian clock and a circadian-regulated daylength measurement mechanism, promotes flowering specifically under LD. The Arabidopsis circadian clock is set by light signals that are perceived by red and far-red light receptors (phytochromes; primarily PHYA and PHYB) and by blue light receptors (cryptochromes; CRY1 and CRY2). The core oscillator is composed of proteins, including CCA1 (CIRCADIAN CLOCK ASSOCIATED1), LHY (LATE ELONGATED HYPOCOTYL), and TOC1 (TIMING OF CAB EXPRESSION1), that are regulated in a negative-feedback loop (Schaffer et al., 1998; Wang and Tobin, 1998; Strayer et al., 2000; Alabadi et al., 2001). The circadian clock takes part in the daylength measurement mechanism that is determined by the regulation of *CONSTANS* (*CO*) gene expression and the light regulation of CO protein stability and activity (Hayama and Coupland, 2004). Induction of flowering in LD is regulated by the gene sequence *GIGANTEA* (*GI*)-*CO*-*FLOWERING LOCUS T* (*FT*). In the leaves, *GI* activates *CO* transcription regardless of photoperiod (Fowler et al., 1999). *CO* transcription is regulated by a

[1] This work was supported by a National Aeronautics and Space Administration West Virginia EPSCoR Research Seed Grant (grant no. 10002987R) and a West Virginia University Summer Undergraduate Research Experience stipend.

[2] Present address: Agronomy Department, Purdue University, 170 South University Street, West Lafayette, IN 47907.

[3] These authors contributed equally to the article.

* Corresponding author; e-mail carina.barth@mail.wvu.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Carina Barth (carina.barth@mail.wvu.edu).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.108.132324

number of factors, including FKF1, GI, and CDF1 (Fowler et al., 1999; Suárez-López et al., 2001; Imaizumi et al., 2003, 2005; Chen and Ni, 2006; Sawa et al., 2007). CO protein is stabilized by PHYA, CRY1, and CRY2, whereas PHYB promotes the degradation of CO (Valverde et al., 2004). Recently, a role of CRY signaling in suppressing COP1-mediated degradation of CO in the dark was also reported (Jang et al., 2008; Liu et al., 2008). CO induces the expression of the floral integrator *FT* (Putterill et al., 2004). Finally, FT protein moves through the phloem to the shoot apex, where FT interacts with the FD transcription factor to induce the expression of the floral identity gene *APETALA1*, which requires the transcription factor *LFY* (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007). Expression of *LFY* is up-regulated by FT (Blazquez, 2005).

Vernalization (i.e. the promotion of flowering by low-temperature treatment) acts, in Arabidopsis, by repression of the floral repressor *FLOWERING LOCUS C* (*FLC*; Martinez-Zapater et al., 1994). Through the action of various genes, including *FCA* (for *FLOWERING TIME CONTROL PROTEIN* α, β, γ, δ), *FLC* is also repressed by an autonomous pathway that induces flowering independently of environmental cues (Quesada et al., 2003; Boss et al., 2004). Finally, GA promotes flowering of Arabidopsis under SD (Wilson et al., 1992; Blazquez et al., 1998; Eriksson et al., 2006). These four flowering pathways converge on the transcriptional regulation of *FT* and *SUPPRESSOR OF CONSTANS1*, which promote *LFY* expression to confer floral identity on developing floral primordia (Corbesier and Coupland, 2005).

Flowering time can also be influenced by other factors. Plants exhibit accelerated flowering in response to shade, drought, low nutrients, decreased light quality, heat, and general oxidative stress (Halliday et al., 1994; Martinez-Zapater et al., 1994; Levy and Dean, 1998; Miller et al., 2007). Furthermore, early work suggested a role of ascorbic acid (AA) in the control of flowering (Chinoy et al., 1957; Hillman, 1962; Bharti and Garg, 1970). Recently, AA-deficient Arabidopsis mutants have been reported to exhibit an altered flowering phenotype. Four AA-deficient mutants, *vtc1*, *vtc2*, *vtc3*, and *vtc4*, were originally isolated by Conklin and coworkers (1996, 2000). These mutants greatly aided in the identification of enzymes that catalyze steps in the predominant AA biosynthetic pathway, which was elucidated by Wheeler et al. (1998). The *VTC1* gene encodes a GDP-Man pyrophosphorylase (Conklin et al., 1999), and the *VTC4* gene encodes an L-Gal-1-P phosphatase (Laing et al., 2004; Conklin et al., 2006). The *VTC2* gene has been cloned (Jander et al., 2002) and has recently been identified as a GDP-L-Gal phosphorylase/L-Gal guanylyl-transferase (Laing et al., 2007; Linster et al., 2007; Smirnoff et al., 2007). The *VTC3* gene has not yet been identified. The point mutations in these *VTC* genes result in the formation of the respective proteins with decreased enzyme activity. Therefore, the *vtc* mutants contain between 20% and 50% of the wild-type AA content (Conklin, 2001). The AA-deficient mutant *vtc1* was reported to flower and senesce before the wild type when grown under LD (Barth et al., 2004; Conklin and Barth, 2004). Interestingly, this mutant and *vtc2* were shown to exhibit delayed flowering and senescence when grown under SD (Pavet et al., 2005). Further support that perturbation of AA levels affects flowering time comes from the observation that artificially increasing the AA content delays flowering in LD-grown Arabidopsis (Attolico and De Tullio, 2006) and *Brassica rapa* (Daniela and De Tullio, 2007) but causes accelerated flowering in *B. rapa* when grown under SD (Daniela and De Tullio, 2007). Based on these reports and current knowledge about mechanisms regulating flowering, several hypotheses were formulated to explain the contrasting flowering phenotypes when AA levels are altered (Barth et al., 2006). However, experimental support for these hypotheses is still lacking.

In order to understand how AA influences flowering time, we exposed the four AA-deficient Arabidopsis mutant lines *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* (Conklin et al., 2000) to SD and LD conditions and assessed their flowering phenotypes. We examined the expression of key regulatory genes in the photoperiodic, autonomous, and GA pathways and generated double mutants of *vtc1-1* and photoperiodic as well as autonomous flowering pathway mutants to investigate whether AA specifically affects any of the known pathways.

RESULTS

AA Deficiency Promotes Flowering and Senescence under Both SD and LD

To assess the flowering phenotype of the wild type and the *vtc* mutants, plants were grown under SD (10 h of light/14 h of dark) and LD (16 h of light/8 h of dark). Vegetative and reproductive growth was assessed over a period of 8 to 11 weeks. Under SD, *vtc1-1*, *vtc3-1*, and *vtc4-1* mutants started flowering at 7 weeks after sowing, whereas the wild type and *vtc2-1* started to produce flowers at 9 weeks after sowing (Fig. 1A). Under LD, all *vtc* mutants started to produce inflorescences about 3 weeks after sowing, whereas the wild type started flowering 4 weeks after sowing (Fig. 1A). It is well established that late-flowering plants form more leaves (Koornneef et al., 1991). Indeed, the early-flowering phenotype of the *vtc* mutants correlated with an approximately 20% decreased number of rosette leaves in comparison with the wild type when grown under SD (Fig. 1, B and C) and LD (Fig. 1, B and D), with the exception of *vtc2-1* under SD. The *vtc2-1* mutant produced significantly fewer leaves than the wild type but more leaves than the other *vtc* mutants under SD (Fig. 1B). However, flower buds appeared at the same time as in the wild type. In addition to the *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* mutants, we also examined the flowering phenotype of

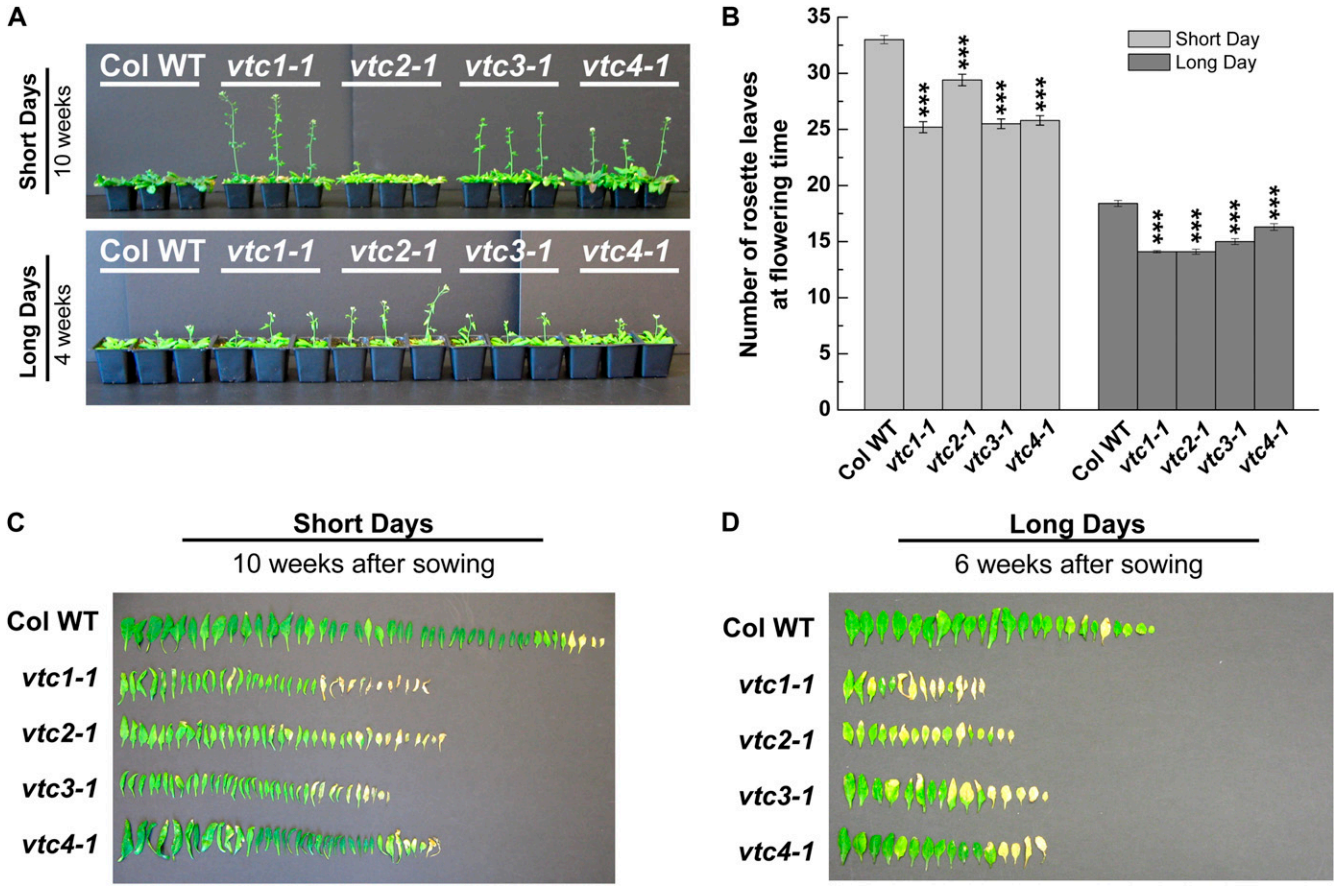

Figure 1. Flowering and senescence phenotypes of wild-type and *vtc* mutant plants. A, Col wild-type (Col WT) and *vtc* mutant plants were grown under SD and LD, and photographs were taken at 10 and 4 weeks after sowing. B, Number of rosette leaves when inflorescences were 1 cm in length. Means ± SE of 10 individual plants are shown. Significant differences in comparison with the wild type are indicated with asterisks: *** $P < 0.001$, by Student's *t* test. C and D, Number of leaves and senescence phenotypes of the wild-type and *vtc* mutant plants grown under SD and LD.

the *vtc2-3* mutant and of *vtc* mutants that were not yet backcrossed to the wild type. These mutant plants also showed early flowering (Supplemental Fig. S1), demonstrating that this phenotype cosegregated with AA deficiency. Based on the early-flowering phenotype of the *vtc* mutants, we hypothesized that the mutants enter senescence before the wild type. In fact, senescence of rosette leaves occurs earlier in the *vtc* mutants than in the wild type irrespective of the photoperiod (Fig. 1, C and D).

In total, our data suggest that AA deficiency causes significantly early flowering and senescence under both SD and LD. It is known that various stress conditions can promote flowering and senescence (Bernier et al., 1993; Martinez-Zapater et al., 1994; Scholl et al., 1998; Kus et al., 2002, Miller et al., 2007). According to the free radical theory of aging, low levels of antioxidants promote the formation of reactive oxygen species (ROS) and thus advance aging and senescence (Harman, 1956). Therefore, we tested whether the *vtc* mutants suffer from oxidative stress by measuring the hydrogen peroxide (H_2O_2) content.

AA Deficiency Results in Slightly Elevated Levels of H_2O_2

Rosette leaves of 3-week-old wild-type and mutant plants grown under SD did not show significant differences in the endogenous H_2O_2 content. However, when plants were 7 weeks old, H_2O_2 levels were about 40% to 90% higher in *vtc1-1* and *vtc2-1* than in the wild type but were unchanged in *vtc3-1* and *vtc4-1* (Supplemental Fig. S2A). In LD-grown mutants, the H_2O_2 content was typically more elevated than in SD-grown mutants, with *vtc1-1* and *vtc2-1* generally exhibiting higher H_2O_2 levels than *vtc3-1* and *vtc4-1*, which correlated with the endogenous amount of leaf AA in the mutants (Supplemental Fig. S3B; Conklin et al., 2000; Conklin, 2001). Note that all *vtc* mutants started flowering at about 3 and 7 weeks after sowing when grown under LD and SD, respectively (except for *vtc2-1*). However, H_2O_2 levels were not significantly elevated in *vtc3-1* and *vtc4-1* compared with the wild type in SD and for *vtc4-1* in LD at the time of flowering, suggesting that the early-flowering phenotype is not

caused just by elevated oxidative stress in the *vtc* mutants. To better define the role of AA deficiency in the control of flowering time, we investigated whether the *vtc* mutants exhibit altered expression of key regulatory genes controlling known flowering pathways.

AA Deficiency Promotes the Expression of Genes Controlling the Circadian Clock and the Photoperiodic Flowering Pathway

The photoperiodic pathway regulates flowering in long photoperiods in the facultative LD plant Arabidopsis (Komeda, 2004; Corbesier and Coupland, 2005; Imaizumi and Kay, 2006). Since the *vtc* mutants had an early-flowering phenotype under LD, we investigated whether transcript levels of circadian clock and photoperiodic pathway genes are heightened in the *vtc* mutants under LD and whether the expression of photoperiodic genes is altered under SD.

Transcription levels were determined in 2- and 5-week old rosette leaves of plants grown under both SD and LD. Neither the wild type nor the *vtc* mutants flowered at these two developmental stages under SD. Under LD, the 2-week-old plants represent the vegetative state, whereas all genotypes were at the reproductive state when they were 5 weeks old (Fig. 1). Transcript levels of *LHY*, a component of the circadian oscillator (Corbesier and Coupland, 2005; Allen et al., 2006), and genes in the photoperiodic pathway, including *GI*, *CO*, and *FT*, are in many cases more than 2-fold up-regulated in the *vtc* mutants under both SD (Fig. 2, A and B) and LD (Fig. 2, C and D). Under the PCR conditions used here, expression of *FT* was not detected in 2-week-old SD-grown plants (Fig. 2A) and expression was low in 5-week-old plants under SD (see different scale on the right *y* axis in Fig. 2B). This was expected, as *vtc* mutants started flowering only 7 weeks after sowing (see above). However, note that mRNA levels in *vtc* mutants are generally elevated at an early developmental stage (2-week-old seedlings), suggesting that AA deficiency constitutively alters the expression of these genes irrespective of the photoperiod and that these flowering time genes act early in development (Araki and Komeda, 1993; Simon et al., 1996).

In summary, the early-flowering phenotype of the *vtc* mutants is associated with significantly higher mRNA levels of circadian clock and photoperiodic pathway genes when plants are grown under either LD or SD. Since the circadian clock regulates *GI* and *CO* transcription, we investigated whether AA deficiency influences circadian rhythms.

AA Deficiency Increases the Amplitude of Transcription of Circadian Oscillator Genes But Does Not Affect Circadian Period

To determine whether AA affects circadian rhythms, we entrained the wild-type and *vtc* mutant plants in day-neutral conditions (12 h of light/12 h of dark). Three weeks after sowing, plants were shifted to constant light. Starting in the last 12-h-light/12-h-dark cycle, expression levels of *LHY*, *TOC1*, *GI*, and *CO* were determined for an additional 48 h under constant light. The oscillator establishes the rhythm of *CO* gene expression, which is mediated by *GI* (Mizoguchi et al., 2005). We investigated whether AA deficiency alters circadian clock period.

In comparison with the wild type, the amplitude of the gene expression rhythm was higher in *vtc1-1* and *vtc3-1*, with the trough level of expression remaining constant but the peak level increased. This expression pattern was most pronounced under free-running (i.e. constant light) conditions (Supplemental Fig. S4). Thus, our data indicate that *LHY*, *TOC1*, *GI*, and *CO* are constitutively up-regulated when endogenous AA levels are low. However, there is no effect on period. Therefore, these data raised the question of what factors would drive this up-regulation. Since light is an important external factor that entrains and maintains circadian rhythms, we investigated whether light input mediated through photoreceptors is altered in the *vtc* mutants compared with the wild type.

AA Deficiency Alters the Expression of Phytochromes and Cryptochromes

Light is perceived by phytochromes and cryptochromes. PHYA, CRY1, and CRY2 positively affect flowering, whereas PHYB represses flowering (Cerdan and Chory, 2003; El-Din El-Assal et al., 2003; Liu et al., 2008). We examined whether *vtc* mutants contain altered transcript levels of *PHYA*, *CRY1*, *CRY2*, and *PHYB*.

Leaves harvested from 5-week-old *vtc* mutants grown under SD and LD generally had significantly higher levels of *PHYA*, *CRY1*, and *CRY2*, whereas *PHYB* mRNA levels were lower in comparison with the wild type (Supplemental Fig. S5). However, differences in transcript levels were in most cases less than 2-fold. This might explain why we did not observe altered hypocotyl growth when plants were grown under dark, white, red, or blue light (Supplemental Fig. S6).

To further investigate a possible effect of AA on PHYB, we tested whether artificially increasing the AA content in the early-flowering *phyB-9* mutant (Columbia-0 [Col] background) by spraying plants with the AA precursor L-Gal would alter flowering time. Although leaf AA levels were significantly elevated in L-Gal-sprayed compared with water-treated *phyB-9* plants (Supplemental Fig. S7A), flowering was not affected (Supplemental Fig. S7, B and C), suggesting that functional PHYB is required for delayed flowering through degradation of CO, as expected (Cerdan and Chory, 2003; El-Din El-Assal et al., 2003). However, this experiment also suggests that early flowering in *phyB* mutants is AA independent. This is supported by the fact that *phyB* mutants are not AA-

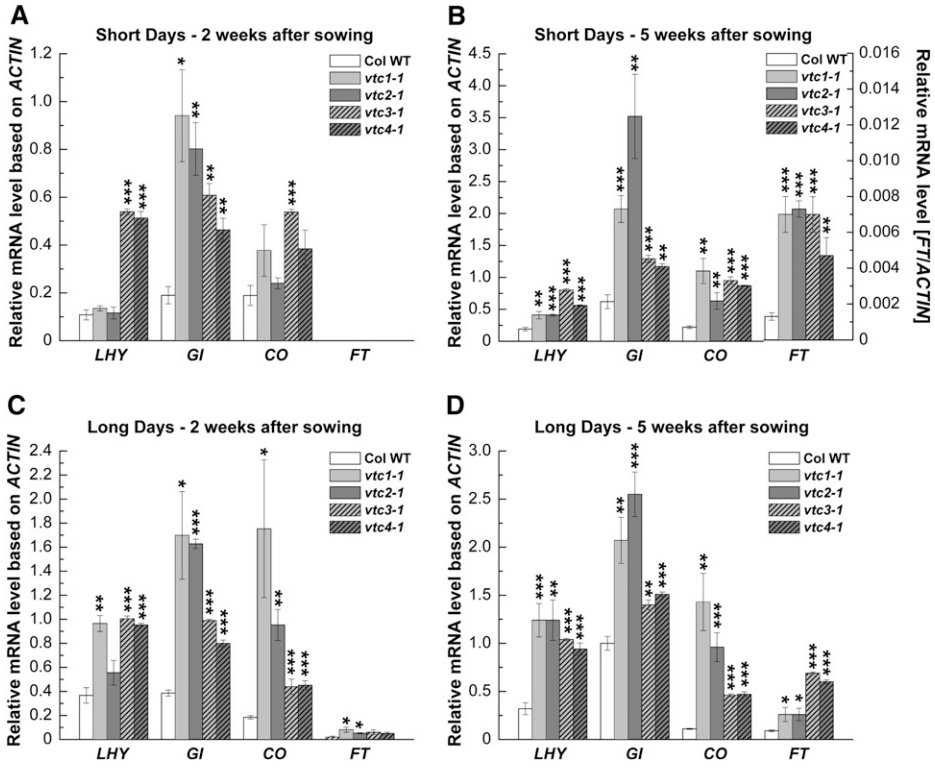

Figure 2. Relative transcript levels, based on *ACTIN*, of circadian clock and photoperiodic pathway genes in Col wild-type (Col WT) and *vtc* mutant plants. Plants were grown under SD (A and B) and LD (C and D). Transcript levels of the circadian clock gene *LHY* and the photoperiodic flowering pathway genes *GI*, *CO*, and *FT* were determined by reverse transcription-PCR from leaf tissue of 2- and 5-week-old plants harvested at 4 h after lights were turned on. Note the different scale to visualize *FT* expression in B. Expression data ± SE of three individual samples per genotype are shown. Significant differences in comparison with the wild type are indicated with asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by Student's *t* test.

deficient. The AA content decreases more rapidly in *phyB* mutants than in the wild type with increasing age, an expected result given that *phyB* mutants are early flowering and senesce after flowering (Supplemental Fig. S7D).

Taken together, our results show that AA deficiency has a modest effect on transcription of red and blue light receptors that influence the expression of genes in the photoperiodic flowering pathway. However, we cannot rule out that AA deficiency influences other flowering pathways, which could contribute to the early-flowering phenotype of the *vtc* mutants.

AA Deficiency Alters the Expression of *FLC*

To test whether the autonomous pathway affects flowering in the *vtc* mutants, we assessed transcript levels of the floral repressor *FLC*, a key integrator in the autonomous pathway. High levels of *FLC* suppress flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). Therefore, we investigated whether *FLC* mRNA levels are lower in *vtc* mutants compared with the wild type. Since FCA is one of several autonomous pathway genes that regulate *FLC*, we also evaluated *FCA* mRNA levels in *vtc* mutants.

The *FCA* transcript is alternatively spliced, resulting in the formation of the four different transcripts, α, β, γ, and δ (Macknight et al., 1997). We used primers that allowed us to amplify a cDNA product generated from the α, γ, and δ transcripts. *FCA*-γ is the only form encoding functional FCA (Macknight et al., 2002). *FCA* mRNA levels were not altered in the *vtc* mutants compared with the wild type when plants were grown under SD (Fig. 3A). *FCA* transcripts were slightly decreased in *vtc1-1* and *vtc2-1*, but not in *vtc3-1* and *vtc4-1*, when grown under LD (Fig. 3B). However, *FLC* transcript levels were approximately 30% lower in SD-grown *vtc1-1*, *vtc3-1*, and *vtc4-1* mutants compared with the wild type, whereas *FLC* mRNA levels were similar in *vtc2-1* and the wild type (Fig. 3A). In contrast, *FLC* transcript levels were more than 2-fold lower ($P < 0.001$) in all *vtc* mutants than in the wild type under LD (Fig. 3B). Under SD, *LFY* mRNA levels were approximately two times higher in the *vtc* mutants than in the wild type, with the exception of *vtc2-1* (Fig. 3A), which is in line with the observed flowering phenotype of the mutants (compare with Fig. 1). Even higher transcript levels of *LFY* were observed under LD (Fig. 3B).

Under noninductive SD conditions, flower initiation depends on the plant hormone GA. The *vtc1* mutant has been reported to contain decreased levels of GA_1 and GA_4 and altered expression of GA biosynthetic genes (Kiddle, 2004; Foyer et al., 2007). We determined expression levels of three AA-dependent GA biosynthetic genes (gibberellin 20-oxidase 1 [*GA20OX1*], gibberellin 3-oxidase 2 [*GA3OX2*], and gibberellin 3-oxidase 4 [*GA3OX4*]; Supplemental Table S2) but could not detect significant differences in expression between the *vtc* mutants and the wild type. Only transcript levels of *GA3OX4*, which could convert GA_{20} into GA_1 and/or GA_4 (Lester et al., 2005; Eriksson et al., 2006),

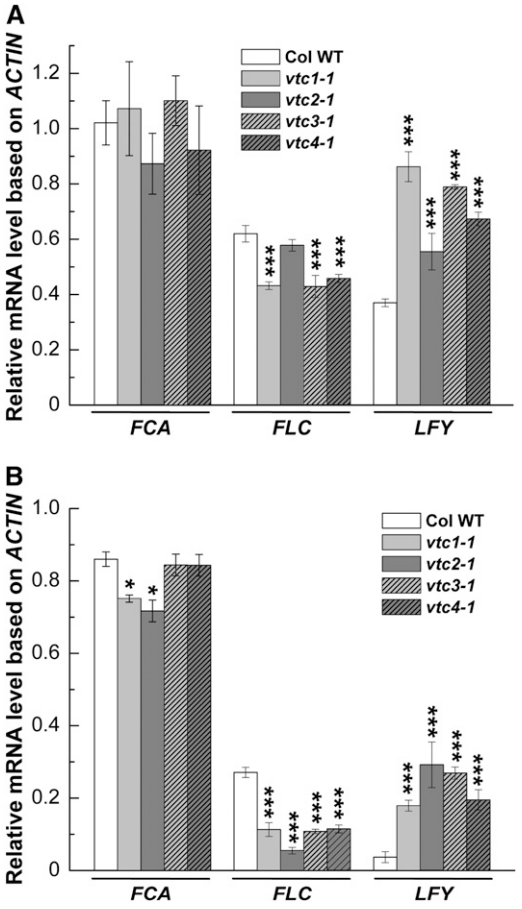

Figure 3. Expression analysis of *FCA*, *FLC*, and *LFY* in Col wild-type (Col WT) and *vtc* mutant plants. Relative transcript levels of *FCA* and *FLC*, which are key regulatory genes in the autonomous pathway, and the floral meristem identity gene *LFY* in inflorescences of 11-week-old plants grown under SD (A) and 5-week-old plants grown under LD (B) are shown. Inflorescence tissues were harvested at 4 h after lights were turned on. Transcript levels were assessed in inflorescences of three individual plants and normalized to *ACTIN*. Means ± SE of three independent replicates are shown. Significant differences in comparison with the wild type are indicated with asterisks: * $P < 0.05$, *** $P < 0.001$, by Student's *t* test.

were higher in *vtc1-1* in comparison with the wild type under SD (Supplemental Fig. S8A) but not under LD (Supplemental Fig. S8B).

In summary, although GA levels were not measured directly, our results indicate that the GA pathway may not or may only partially contribute to the early-flowering phenotype in the *vtc* mutants. To further investigate the role of AA on flowering time, we analyzed the flowering phenotype and expression of genes in the photoperiodic and autonomous pathways in plants containing artificially elevated levels of AA.

Artificially Increasing the Level of AA Delays Flowering and Senescence

We presented evidence that low levels of AA confer early flowering that correlates with an up-regulation of circadian clock and photoperiodic pathway genes and a down-regulation of *FLC* mRNA. Conversely, we asked whether flowering and senescence can be delayed when we artificially increase the endogenous AA content and whether genes that are up-regulated in the *vtc* mutants in comparison with the wild type are down-regulated in plants containing elevated levels of AA. To elevate AA levels, plants were sprayed with L-Gal, an intermediate in the AA biosynthetic pathway (Wheeler et al., 1998).

In comparison with plants sprayed with water, L-Gal treatment resulted in an approximately 2-fold increase in the total AA content in LD-grown plants (Fig. 4A). Flowering of L-Gal-sprayed plants grown under LD was delayed by about 5 d in comparison with plants sprayed with water (Fig. 4B). This is supported by the fact that L-Gal-sprayed plants developed 17 ± 0.4 (SE) rosette leaves, whereas water-sprayed plants formed only 13 ± 0.4 rosette leaves ($n = 16$ in both cases; Fig. 4C). The delayed-flowering phenotype was accompanied by delayed senescence (Fig. 4C). In comparison with the water-sprayed plants, we detected lower mRNA levels of *PHYA*, *CRY1*, *CRY2*, *LHY*, *GI*, *CO*, *FT*, and *LFY* in L-Gal-sprayed plants, whereas transcript levels of *PHYB* and *FLC* were increased and those of *FCA* were not changed (Fig. 4D). Similar results were found in L-Gal- and water-treated wild-type plants grown under SD (Supplemental Fig. S9).

In summary, the results presented in Figure 4 and Supplemental Figure S9 are in accordance with data obtained for the AA-deficient *vtc* mutants. Furthermore, these data support our observation that alterations in the leaf AA content significantly affect the onset of flowering that correlates with transcriptional changes of genes in the photoperiodic, autonomous, and light perception pathways. Low levels of AA promote flowering and senescence, whereas high levels of AA delay flowering and senescence irrespective of the photoperiod. The data presented above suggest that AA acts upstream of pathways that control flowering. To test this, we crossed the early-flowering mutant *vtc1-1* to photoperiodic and autonomous pathway mutants, which are delayed in flowering.

vtc1-1 gi-1, *vtc1-1 co-2*, *vtc1-1 ft-1*, and *vtc1-1 fca-1* Double Mutants Exhibit Delayed Flowering Despite Their AA Deficiency

To test whether AA plays a specific role in the photoperiodic and/or the autonomous pathways, we crossed *vtc1-1* to the late-flowering photoperiodic pathway mutants *gi-1*, *co-2*, and *ft-1* and to the autonomous pathway mutant *fca-1*, which is also delayed in flowering (Koornneef et al., 1991; Putterill et al., 1995; Macknight et al., 1997; Fowler et al., 1999; Suárez-López et al., 2001). If AA specifically acts in the autonomous pathway, *vtc1-1 gi-1*, *vtc1-1 co-2*, and *vtc1-1 ft-1* double mutants should exhibit a promotion of flowering compared with *gi-1*, *co-2*, and *ft-1* single

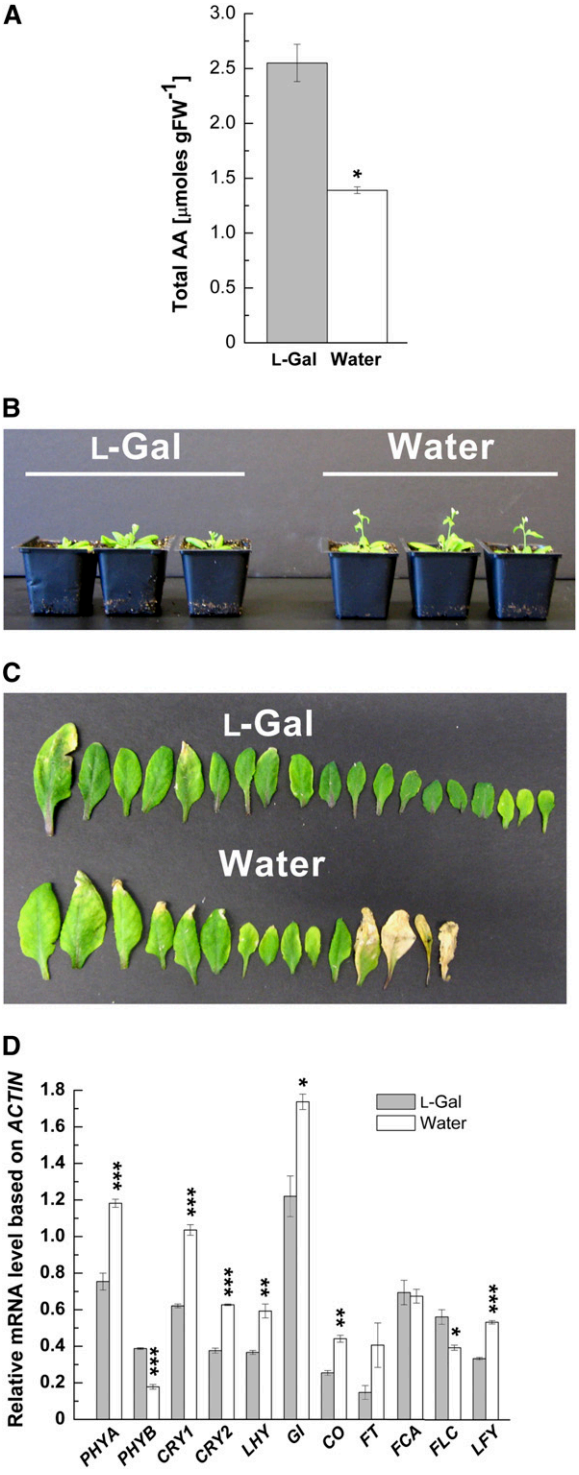

Figure 4. Effects of L-Gal treatment on flowering time in LD-grown plants. A, Total AA content in 5-week-old Col wild-type plants sprayed with L-Gal or water. FW, Fresh weight. B, Flowering phenotype of L-Gal- and water-treated plants. C, Number of leaves and senescence phenotype of L-Gal- and water-sprayed plants. D, Expression analysis of photoreceptor (*PHYA*, *PHYB*, *CRY1*, and *CRY2*), circadian clock (*LHY*), photoperiodic pathway (*GI*, *CO*, and *FT*), autonomous (*FCA* and *FLC*), and floral meristem identity (*LFY*) genes in plants treated with L-Gal or water. Leaf and inflorescence tissues of 5-week-old plants were harvested at 4 h after lights were turned on. To assess the expression of *FT*, 33 PCR amplification cycles were run. Transcript levels were based on *ACTIN*. Results represent means ± SE of three independent replicates. Significant differences between L-Gal and water treatments are indicated with asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by Student's *t* test.

mutants due to the activity of the autonomous pathway in *vtc1-1* (Fig. 3). Likewise, *vtc1-1 fca-1* double mutants are expected to exhibit earlier flowering than *fca-1* single mutants due to the enhanced activity of the photoperiodic pathway measured in *vtc1-1* (Fig. 2; Supplemental Fig. S4).

All double mutants displayed a delayed-flowering phenotype despite their AA deficiency (Fig. 5A). Note that *vtc1-1 fca-1* has approximately 70% of the Col/Landsberg *erecta* (L*er*) wild-type AA content, suggesting that *fca-1* can partially suppress the *vtc1-1* mutation. All homozygous double mutants had a similar number of rosette leaves as the *gi-1*, *co-2*, *ft-1*, and *fca-1* single mutants, whereas *vtc1-1* produced fewer rosette leaves than the Col wild type under LD (Fig. 5B) and SD (Supplemental Fig. S10). Note that the SE bars for both the AA content and flowering time data in the Col/L*er* wild type and in the double mutants of two different backgrounds are comparable to the SE bars of the respective wild-type controls, suggesting low genetic variability in the pools of plants used for the experiments.

In summary, our genetic analysis demonstrates that the *gi-1*, *co-2*, *ft-1*, and *fca-1* mutations are all epistatic to *vtc1-1*, suggesting that *GI*, *CO*, *FT*, and *FCA* play roles in the promotion of flowering of *vtc1-1*. However, AA does not appear to have a specific role in any of the known flowering pathways.

DISCUSSION

Recent publications reported contrasting flowering and senescence phenotypes of the *vtc1* and *vtc2* mutants, exhibiting delayed and early flowering/senescence under SD (Pastori et al., 2003; Pavet et al., 2005) and LD (Barth et al., 2004; Conklin and Barth, 2004), respectively. In order to understand how AA influences flowering time, we examined the flowering phenotype of the wild type in comparison with four AA-deficient mutant lines grown under SD and LD.

We found that all four *vtc* mutants, with the exception of *vtc2-1* grown under SD, exhibit early flowering irrespective of the photoperiod (Fig. 1). This is in contrast to findings by Pastori et al. (2003) and Pavet et al. (2005). Investigation of *vtc* mutants, which were not yet backcrossed to the wild type, also revealed an early-flowering phenotype (Supplemental Fig. S1), suggesting that the observed differences are not due to the presence of additional mutations and are related to AA deficiency. Therefore, the reason for the observed contrasting flowering phenotypes is unclear at present. Growth conditions, including nutrient supply

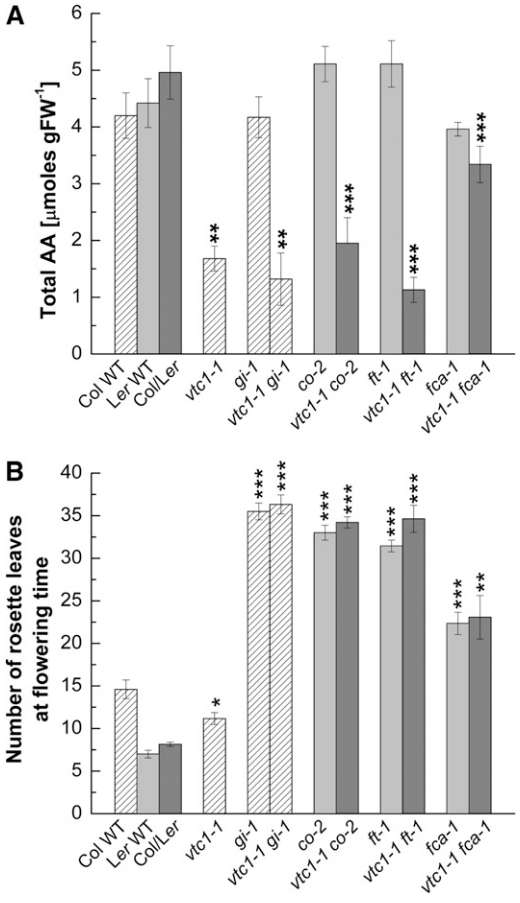

Figure 5. Effects of the *vtc1-1* mutation on flowering time and AA content in the background of photoperiodic and autonomous pathway mutants. Plants were grown under LD. A, Total AA content in 3-week-old wild-type (WT) controls and single and double mutants. Means ± SE of three to six independent replicates per genotype are shown. FW, Fresh weight. B, Total rosette leaf number of all genotypes at flowering time. Results depict means ± SE of nine to 17 independent plants per genotype. Shading patterns indicate plants of the same genetic background, allowing for easier statistical comparison of single and double mutants with their respective wild-type controls. Asterisks denote significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by Student's *t* test.

and light intensity, may alter flowering time and could account for the differences in the time of flowering. For example, the lower light intensity used in the experiments reported here (160 μmol photons m^{-2} s^{-1}) under both SD and LD conditions compared with the higher one used by the Pastori and Pavet groups (250 μmol photons m^{-2} s^{-1}) under SD could differentially affect AA pool size and redox status. Higher light intensities increase leaf ascorbate concentration (Smirnoff, 2000; Bartoli et al., 2006; Dowdle et al., 2007), whereas changes in photoperiods only affect AA pool size slightly or not at all (Queval et al., 2007; Supplemental Fig. S3). Thus, one would expect delayed flowering in plants grown at higher light intensities. It would hence be worthwhile to investigate the flowering time of *vtc* mutants grown under different light intensities.

Finally, based on rosette leaf number and transcript levels of photoperiodic pathway genes (Fig. 2, A and B), *vtc2-1* would be considered early flowering under SD. However, flower buds emerged at the same time as in the wild type, which correlates with the expression of the autonomous pathway gene *FLC* (Fig. 3). The reason for the different response of *vtc2-1* under SD is unclear at present. Nevertheless, we provide evidence that alterations in the AA content have a significant effect on flowering time.

AA Influences Flowering Time through a Mechanism That Is Independent of Its Antioxidant Activity

Early flowering in AA-deficient *vtc* mutants (Fig. 1) and late flowering in plants with artificially elevated AA levels (Fig. 4; Supplemental Fig. S9) could simply be explained by the antioxidant function of AA to scavenge ROS. Low levels of AA, as in the *vtc* mutants (Supplemental Fig. S3; Conklin et al., 2000), would be expected to result in elevated levels of ROS. Indeed, the *vtc* mutants contain elevated H_2O_2 levels (Supplemental Fig. S2), suggesting that they are suffering some oxidative stress under optimal growth conditions, ultimately resulting in early flowering/senescence. However, our data suggest that the early-flowering phenotype cannot be solely attributed to increased oxidative stress in the *vtc* mutants. This conclusion is supported by the following facts. First, oxidative stress in the *vtc* mutants compared with the wild type is generally higher under LD than SD, with little effect on *vtc3-1* and *vtc4-1* grown at SD. However, the flowering phenotype of the *vtc* mutants does not correlate with the endogenous H_2O_2 content (compare Fig. 1 and Supplemental Fig. S2). For example, the *vtc3-1* and *vtc4-1* mutants generally exhibit lower or similar levels of H_2O_2 than the wild type (Supplemental Fig. S2), but the mutants still exhibit an early-flowering phenotype (Fig. 1). Second, in comparison with the wild type, the difference in leaf number produced in the *vtc* mutants is similar under SD and LD (*vtc* mutants formed approximately 20% fewer leaves than the wild type under SD and LD, except for *vtc2-1* grown under SD; Fig. 1B). Third, Arabidopsis mutants lacking cytosolic ascorbate peroxidase and exhibiting constitutive accumulation of H_2O_2 under optimal growth conditions show delayed flowering under LD and constant light (Pnueli et al., 2003). It should be mentioned, however, that some double mutants deficient in cytosolic and thylakoid ascorbate peroxidase suffering oxidative stress exhibit early flowering (Miller et al., 2007). Fourth, double mutants of *vtc1-1* and flowering time mutants are AA deficient (Fig. 5A) but have delayed flowering (Fig. 5B). In summary, our data demonstrate that AA influences flowering in a photoperiod-independent manner through factors (discussed in detail below) other than or perhaps in addition to ROS and/or redox changes. Therefore, expression and genetic analyses were con-

ducted to establish whether AA specifically acts in known flowering time pathways.

AA Does Not Seem to Act Specifically in Any of the Known Flowering Pathways and May Play a General Role in Responding to Environmental Signals

Data presented in this study show that AA has a significant effect on flowering time and that alterations in the AA content confer differential gene expression of all genes in known pathways. The alterations in gene expression patterns are consistent with the observed flowering phenotypes (Figs. 2–4; Supplemental Figs. S4, S5, S8, and S9). In fact, the expression patterns in the *vtc* mutants and in wild-type plants with artificially altered AA levels would fit the external coincidence model (Yanovsky and Kay, 2003; Hayama and Coupland, 2004; Putterill et al., 2004; Saunders, 2005; Imaizumi and Kay, 2006; Kobayashi and Weigel, 2007) as well as the function of phytochromes and cryptochromes in the regulation of CO (Reed et al., 1993; Guo et al., 1998; Mockler et al., 1999, 2003; Yang et al., 2000; Cerdan and Chory, 2003; Valverde et al., 2004). AA deficiency, however, does not affect the circadian clock despite enhancing the expression of clock genes, including *LHY* (Fig. 2; Supplemental Fig. S4). Early flowering may be promoted through enhanced expression of *GI*, which promotes the expression of *LHY/CCA1*, *CO*, and *FT*, conferring early flowering. Such a phenomenon has been reported recently (Oda et al., 2004). Oda and coworkers (2004) suppressed the *PHYTOCHROME INTERACTING FACTOR3* gene, resulting in higher *CO* and *FT* mRNA levels, and thus in early flowering, under LD without affecting circadian period.

Taken together, our expression and genetic analyses do not support a specific role of AA in the known flowering pathways (Fig. 5, B and C). The mechanism through which AA influences flowering time, therefore, remains unclear. Instead, the picture that emerges is that alterations in the AA content confer perturbations in plant metabolism and gene expression that indirectly affect flowering. The observed gene expression differences cause a variety of pleiotropic effects in the *vtc* mutants. This may be explained by the fact that, in addition to its function as an antioxidant, AA also serves as an essential cofactor for a variety of enzymes. For example, AA is required for the biosynthesis of the plant hormones abscisic acid, GA, and ethylene (Arrigoni and De Tullio, 2000, 2002). Previous studies have demonstrated a high content of abscisic acid (Pastori et al., 2003; S.O. Kotchoni and C. Barth, unpublished data) but low levels of GA (Kiddle, 2004; Foyer et al., 2007) in *vtc1*. Therefore, one would expect the late-flowering phenotype of the *vtc* mutants (Barth et al., 2006), as reported by Pastori et al. (2003) and Pavet et al. (2005).

In addition to changes in the abscisic acid and GA contents, low levels of AA promote the accumulation of the phytoalexin camalexin (Colville and Smirnoff, 2008) and salicylic acid (Barth et al., 2004; M. Mukherjee and C. Barth, unpublished data), which has been demonstrated to also influence flowering time (Martinez et al., 2004). It was also shown that de novo AA synthesis increases in response to methyl jasmonate (Sasaki-Sekimoto et al., 2005; Wolucka et al., 2005). Glutathione (Pavet et al., 2005) and α-tocopherol (Kanwischer et al., 2005) have been reported to be slightly elevated in *vtc1* or unchanged in *vtc* mutants (Colville and Smirnoff, 2008). Consequently, alterations in the AA pool size result in hormonal changes, conferring altered gene expression, leading to a variety of phenotypes in the *vtc* mutants. These include altered flowering (Pastori et al., 2003; Conklin and Barth, 2004; this study), enhanced resistance to virulent pathogens (Barth et al., 2004; Pavet et al., 2005), altered primary root development (Olmos et al., 2006), changes in energy status (Wormuth et al., 2006), altered heat tolerance (Larkindale et al., 2005), increased sensitivity to salt stress (Huang et al., 2005), altered cell wall biosynthesis (Lukowitz et al., 2001), and sensitivity to ozone (Conklin et al., 1996, 2000). Whereas some of those phenotypes can clearly be attributed to the lack of antioxidant capacity of the *vtc* mutants, some phenotypes cannot simply be explained by elevated ROS levels and altered redox status in the mutants. In fact, the ascorbate oxidation state does not vary in the *vtc* mutants (Conklin et al., 2000; Colville and Smirnoff, 2008; M. Mukherjee and C. Barth, unpublished data). This study provides evidence that the AA pool size affects the regulation of growth and developmental and defense responses. Thus, it is possible that AA levels serve as an internal signal that helps properly poise plants to respond to environmental signals and facilitates the adjustment of plant development, including the transition from the vegetative to the reproductive phase. To evaluate this possibility, future experiments will have to address how plants perceive AA, how the endogenous AA content is regulated, and how plants translate alterations in the AA pool size into developmental adjustments.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type ecotype Col and previously described Arabidopsis mutants *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* (kindly provided by P. Conklin; Conklin et al., 1996, 2000; Conklin, 2001) were grown in a growth chamber (Percival). Mutant plants were backcrossed to Col wild type four times (*vtc1-1*), three times (*vtc3-1*), or two times (*vtc2-1* and *vtc4-1*). The flowering time mutants *gi-1* (CS3123), *co-2* (CS175 and CS55), *ft-1* (CS56), and *fca-1* (CS167) and the wild type L*er* (CS20) were obtained from the Arabidopsis Biological Resource Center Stock Center. Plants were grown on soil (Metromix 360; BFG Supplies) in replicate flats containing 32 inserts with wild-type controls and mutants always present on the same flat. Temperature in the chamber was 23°C at day and at night. Plants were grown under LD (16 h of light/8 h of dark, growth chamber lights turned on at 6:00 AM and turned off at 10:00 PM) and SD (10 h of light/14 of h dark, growth chamber lights turned on at 6:00 AM and turned off at 4:00 PM) at a light intensity of 160 μmol photons m^{-2} s^{-1} (fluorescent bulbs).

Two-week-old plants were harvested by pooling aboveground tissue of seedlings. When plants were 3 weeks old, rosette leaves of similar age were marked using a soft marker. Rosette leaves were pooled from two to four individual plants. Plants were only used once for tissue harvest to avoid gene

expression alterations due to wound effects. In all cases, plant tissue was collected at 4 h after growth chamber lights were turned on, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis.

Generation and Identification of Double Mutants

The *vtc1-1* mutant (Col background; Conklin et al., 2000) was crossed with *gi-1* (Col background; Fowler et al., 1999), *co-2* (L*er* background; Putterill et al., 1995), *ft-1* (L*er* background; Kardailsky et al., 1999), and *fca-1* (L*er* background; Macknight et al., 1997). F1 progeny of the crosses were allowed to self. F2 progeny were screened for AA deficiency (Conklin et al., 2000), and DNA was extracted from progeny that scored as AA deficient. Per plant, two PCR procedures were carried out to identify homozygous *vtc1-1 gi-1*, *vtc1-1 co-2*, *vtc1-1 ft-1*, and *vtc1-1 fca-1* double mutants using the primers listed in Supplemental Table S1. F3 seeds from homozygous double mutants were used for experiments. The individual Col and L*er* wild types and the Col × L*er* crosses were used as controls. To minimize genetic variability, resulting from crossing two different backgrounds, seeds were pooled from 12 different crossing events of the Col and L*er* wild-type controls (Col × L*er*; Miller et al., 2007), and at least three independent double mutants (except for *vtc1-1 co-2*) were evaluated for flowering time.

Measurement of H_2O_2 Content

Freshly harvested rosette leaves (approximately 0.1 g) were incubated by shaking (250 rpm) in 3 mL of 25 mM phosphate buffer, pH 7.0, containing 0.05% guaiacol (Sigma) and 2.5 units mL^{-1} horseradish peroxidase (Sigma) in the dark at 25°C for 2 h. Absorbance of the solution was measured at 450 nm as described (Von Tiedemann, 1997). H_2O_2 concentrations were determined using a H_2O_2 standard curve, containing 5, 10, 25, 50, 75, 100, or 150 μM H_2O_2 (Sigma) as described (Kotchoni et al., 2006).

Determination of Flowering Time

Flowering time was assessed by counting the number of rosette leaves when flower bolts were 1 cm in length or when floral buds were visible at the center of the rosette.

Circadian Rhythm Experiments

Wild-type and *vtc* mutant plants were entrained in day-neutral (12 h of light/12 h of dark) conditions for 3 weeks and then subjected to constant light for 48 h. Rosette leaves were harvested starting in the last 12-h-light/12-h-dark cycle (chamber lights turned on at 6:00 AM and turned off at 6:00 PM), beginning 1 h after lights were turned on. Tissue was harvested every 4 h in the last dark/light cycle, and tissue collection continued for another 48 h under constant light. Temperature in the growth chamber was 23°C throughout the experiment. The Percival growth chambers used for these experiments were programmed for temperature and photoperiod. A 12-h-light/12-h-dark photoperiod program with constant temperature was created for the first 3 weeks of growth. The program was then switched to constant light for another 48 h.

Exogenous Application of L-Gal

Wild-type plants were grown under SD and LD as described above. Plants were sprayed every other day with either water or 10 mM L-Gal. Spraying started when plants were 8 d old and continued until plants finished their life cycle. Tissue of 5- and 13-week-old plants grown under SD and LD was harvested for gene expression analyses.

Measuring AA Content

Leaf AA content was determined in whole 3-week-old rosettes using the ascorbate oxidase assay (Conklin et al., 1997) and the iron reduction assay (Dowdle et al., 2007).

RNA Isolation, cDNA Synthesis, and Gene Expression Analysis

Total RNA from rosette leaves and inflorescences was extracted using Tri-Reagent (Molecular Research Center). Five microliters of total RNA was subjected to reverse transcription using a first-strand cDNA synthesis kit (Invitrogen) and 10 pg of oligo(dT) primers. Two micrograms of cDNA was utilized for PCR using gene-specific primers (Supplemental Table S2), running 20 or 25 amplification cycles (linear range of amplification) unless otherwise noted. The linear range of amplification was determined by running increasing cycle numbers and analyzing the amount of cDNA fragments. PCR fragments were separated on 1% agarose gels containing ethidium bromide. Band intensities were quantified with ImageQuant 5.0 (Amersham Biosciences). A cDNA fragment generated from *ACTIN* served as an internal control.

Statistical Analyses

Data are expressed as mean values ± SE. Experiments were repeated at least three times. *P* values were determined by Student's *t* test analysis.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Flowering phenotype of Col wild-type and *vtc* mutant plants under LD.

Supplemental Figure S2. H_2O_2 content in Col wild-type and *vtc* mutant plants grown under SD and LD.

Supplemental Figure S3. Total AA content in Col wild-type and *vtc* mutant plants grown under SD and LD.

Supplemental Figure S4. Circadian rhythms of clock and photoperiod pathway genes in Col wild-type and *vtc* mutant plants.

Supplemental Figure S5. Expression analysis of phytochrome and cryptochrome genes in Col wild-type and *vtc* mutant plants.

Supplemental Figure S6. Hypocotyl length in Col wild type, L*er* wild type, *vtc*, and phytochrome and cryptochrome mutants.

Supplemental Figure S7. Effect of L-Gal on flowering time in *phyB-9* and developmental changes in the AA content in wild-type, *vtc1-1*, and *phyB* plants grown under LD.

Supplemental Figure S8. Expression analysis of GA 3-oxidase 4 in Col wild-type and *vtc* mutant plants.

Supplemental Figure S9. Effect of L-Gal on flowering time in SD-grown plants.

Supplemental Figure S10. Flowering phenotype of wild-type controls, *vtc1-1*, and flowering time single mutants and double mutants with *vtc1-1* under SD.

Supplemental Table S1. Sequences of oligonucleotide primers used for mutant identification.

Supplemental Table S2. Sequences of oligonucleotide primers used for gene expression analysis.

ACKNOWLEDGMENTS

We thank Patricia Conklin for providing seeds of the *vtc* mutants and Georg Jander as well as Dale Karlson for helpful comments on the manuscript.

Received November 8, 2008; accepted November 17, 2008; published November 21, 2008.

LITERATURE CITED

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309:** 1052–1056

Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, Kay SA (2001)

Reciprocal regulation between *TOC1* and *LHY/CCA1* within the *Arabidopsis* circadian clock. Science **293:** 880–883

Allen T, Koustenis A, Theodorou G, Somers DE, Kay SA, Whitelam GC, Devlin PF (2006) *Arabidopsis* FHY3 specifically gates phytochrome signaling to the circadian clock. Plant Cell **18:** 2506–2516

Araki T, Komeda Y (1993) Analysis of the role of the late-flowering locus, *Gl*, in the flowering of *Arabidopsis thaliana*. Plant J **3:** 231–239

Arrigoni O, De Tullio MC (2000) The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions. J Plant Physiol **157:** 481–488

Arrigoni O, De Tullio MC (2002) Ascorbic acid: much more than just an antioxidant. Biochim Biophys Acta **1569:** 1–9

Attolico AD, De Tullio MC (2006) Increased ascorbate content delays flowering in long-day grown *Arabidopsis thaliana* (L.). Heynh. Plant Physiol Biochem **44:** 462–466

Barth C, De Tullio M, Conklin PL (2006) The role of ascorbic acid in the control of flowering time and the onset of senescence. J Exp Bot **57:** 1657–1665

Barth C, Moeder W, Klessig DF, Conklin PL (2004) The timing of senescence and response to pathogens is altered in the ascorbate-deficient Arabidopsis mutant vitamin c-1. Plant Physiol **134:** 1784–1792

Bartoli CG, Yu J, Gomez F, Fernandez L, McIntosh L, Foyer CH (2006) Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in *Arabidopsis thaliana* leaves. J Exp Bot **57:** 1621–1631

Bernier G, Havelange A, Houssa C, Petitjean A, Lejeune P (1993) Physiological signals that induce flowering. Plant Cell **5:** 1147–1155

Bharti S, Garg OP (1970) Changes in the ascorbic acid content of the lateral buds of soybean in relation to flower induction. Plant Cell Physiol **11:** 723–727

Blazquez MA (2005) Plant science. The right time and place for making flowers. Science **309:** 1024–1025

Blazquez MA, Green R, Nilsson O, Sussman MR, Weigel D (1998) Gibberellins promote flowering of *Arabidopsis* by activating the *LEAFY* promoter. Plant Cell **10:** 791–800

Boss PK, Bastow RM, Mylne JS, Dean C (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. Plant Cell (Suppl) **16:** S18–S31

Cerdan PD, Chory J (2003) Regulation of flowering time by light quality. Nature **423:** 881–885

Chen M, Ni M (2006) RFI2, a RING-domain zinc finger protein, negatively regulates *CONSTANS* expression and photoperiodic flowering. Plant J **46:** 823–833

Chinoy JJ, Nanda KK, Garg OP (1957) Effect of ascorbic acid on growth and flowering of *Trigonella foenum-graecum* and *Brassica chinensis*. Physiol Plant **10:** 869–876

Colville L, Smirnoff N (2008) Antioxidant status, peroxidase activity, and PR protein transcript levels in ascorbate-deficient *Arabidopsis thaliana vtc* mutants. J Exp Bot **59:** 3857–3868

Conklin PL (2001) Recent advances in the role and biosynthesis of ascorbic acid in plants. Plant Cell Environ **24:** 383–394

Conklin PL, Barth C (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ **27:** 959–971

Conklin PL, Gatzek S, Wheeler GL, Dowdle J, Raymond MJ, Rolinski S, Isupov M, Littlechild JA, Smirnoff N (2006) *Arabidopsis thaliana VTC4* encodes L-galactose-1-P phosphatase, a plant ascorbic acid biosynthetic enzyme. J Biol Chem **281:** 15662–15670

Conklin PL, Norris SR, Wheeler GL, Williams EH, Smirnoff N, Last RL (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. Proc Natl Acad Sci USA **96:** 4198–4203

Conklin PL, Pallanca JE, Last RL, Smirnoff N (1997) L-Ascorbic acid metabolism in the ascorbate-deficient Arabidopsis mutant *vtc1*. Plant Physiol **115:** 1277–1285

Conklin PL, Saracco SA, Norris SR, Last RL (2000) Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants. Genetics **154:** 847–856

Conklin PL, Williams EH, Last RL (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. Proc Natl Acad Sci USA **93:** 9970–9974

Corbesier L, Coupland G (2005) Photoperiodic flowering of Arabidopsis: integrating genetic and physiological approaches to characterization of the floral stimulus. Plant Cell Environ **28:** 54–66

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, et al (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science **316:** 1030–1033

Daniela AA, De Tullio M (2007) Contrasting effects of increased ascorbate content on growth and development of long-day and short-day grown *Brassica rapa*. Caryologia **60:** 185–187

Dowdle J, Ishikawa T, Gatzek S, Rolinski S, Smirnoff N (2007) Two genes in *Arabidopsis thaliana* encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. Plant J **52:** 673–689

El-Din El-Assal S, Alonso-Blanco C, Peeters AJ, Wagemaker C, Weller JL, Koornneef M (2003) The role of cryptochrome 2 in flowering in Arabidopsis. Plant Physiol **133:** 1504–1516

Eriksson S, Bohlenius H, Moritz T, Nilsson O (2006) GA4 is the active gibberellin in the regulation of *LEAFY* transcription and *Arabidopsis* floral initiation. Plant Cell **18:** 2172–2181

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J (1999) *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J **18:** 4679–4688

Foyer CH, Kiddle G, Verrier P (2007) Transcriptional profiling approaches to understanding how plants regulate growth and defence: a case study illustrated by analysis of the role of vitamin C. EXS **97:** 55–86

Garner WW, Allard HA (1920) Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. J Agric Res **18:** 553–606

Garner WW, Allard HA (1923) Further studies on photoperiodism, the response of plants to relative length of day and night. J Agric Res **23:** 871–920

Guo H, Yang H, Mockler TC, Lin C (1998) Regulation of flowering time by Arabidopsis photoreceptors. Science **279:** 1360–1363

Halliday KJ, Koornneef M, Whitelam GC (1994) Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of *Arabidopsis thaliana* L. to low red/far-red ratio. Plant Physiol **104:** 1311–1315

Harman D (1956) Aging: a theory based on free radical and radiation chemistry. J Gerontol **11:** 298–300

Hayama R, Coupland G (2004) The molecular basis of diversity in the photoperiodic flowering responses of *Arabidopsis* and rice. Plant Physiol **135:** 677–684

Hillman WS (1962) Experimental control of flowering in *Lemna*. IV. Inhibition of photoperiodic sensitivity by copper. Am J Bot **49:** 892–897

Huang C, He W, Guo J, Chang X, Su P, Zhang L (2005) Increased sensitivity to salt stress in an ascorbate-deficient Arabidopsis mutant. J Exp Bot **56:** 3041–3049

Imaizumi T, Kay SA (2006) Photoperiodic control of flowering: not only by coincidence. Trends Plant Sci **11:** 550–558

Imaizumi T, Schultz TF, Harmon FG, Ho LA, Kay SA (2005) FKF1 F-box protein mediates cyclic degradation of a repressor of *CONSTANS* in *Arabidopsis*. Science **309:** 293–297

Imaizumi T, Tran HG, Swartz TE, Briggs WR, Kay SA (2003) FKF1 is essential for photoperiodic-specific light signalling in *Arabidopsis*. Nature **426:** 302–306

Jack T (2004) Molecular and genetic mechanisms of floral control. Plant Cell (Suppl) **16:** S1–S17

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL (2002) Arabidopsis map-based cloning in the post-genome era. Plant Physiol **129:** 440–450

Jang S, Marchal V, Panigrahi KC, Wenkel S, Soppe W, Deng XW, Valverde F, Coupland G (2008) Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J **27:** 1277–1288

Kanwischer M, Porfirova S, Bergmuller E, Dormann P (2005) Alterations in tocopherol cyclase activity in transgenic and mutant plants of Arabidopsis affect tocopherol content, tocopherol composition, and oxidative stress. Plant Physiol **137:** 713–723

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer FT. Science **286:** 1962–1965

Kiddle G (2004) The role of ascorbate in plant defence and development. PhD thesis. University of Bristol, Bristol, UK

Klebs G (1913) Über das Verhältnis der Aussenwelt zur Entwicklung der Pflanze. Heidelberger Academie der Wissenschaften **5:** 1–47

Kobayashi Y, Weigel D (2007) Move on up, it's time for change: mobile signals controlling photoperiod-dependent flowering. Genes Dev **21:** 2371–2384

Komeda Y (2004) Genetic regulation of time to flower in *Arabidopsis thaliana*. Annu Rev Plant Biol **55:** 521–535

Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol Gen Genet **229:** 57–66

Kotchoni SO, Kuhns C, Ditzer A, Kirch HH, Bartels D (2006) Overexpression of different aldehyde dehydrogenase genes in *Arabidopsis thaliana* confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress. Plant Cell Environ **29:** 1033–1048

Kus JV, Zaton K, Sarkar R, Cameron RK (2002) Age-related resistance in Arabidopsis is a developmentally regulated defense response to *Pseudomonas syringae*. Plant Cell **14:** 479–490

Laing WA, Bulley S, Wright M, Cooney J, Jensen D, Barraclough D, MacRae E (2004) A highly specific L-galactose-1-phosphate phosphatase on the path to ascorbate biosynthesis. Proc Natl Acad Sci USA **101:** 16976–16981

Laing WA, Wright MA, Cooney J, Bulley SM (2007) The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. Proc Natl Acad Sci USA **104:** 9534–9539

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol **138:** 882–897

Lester DR, Phillips A, Hedden P, Andersson I (2005) Purification and kinetic studies of recombinant gibberellin dioxygenases. BMC Plant Biol **5:** 19

Levy YY, Dean C (1998) The transition to flowering. Plant Cell **10:** 1973–1990

Linster CL, Gomez TA, Christensen KC, Adler LN, Young BD, Brenner C, Clarke SG (2007) Arabidopsis *VTC2* encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. J Biol Chem **282:** 18879–18885

Liu LJ, Zhang YC, Li QH, Sang Y, Mao J, Lian HL, Wang L, Yang HQ (2008) COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in *Arabidopsis*. Plant Cell **20:** 292–306

Lukowitz W, Nickle TC, Meinke DW, Last RL, Conklin PL, Somerville CR (2001) *Arabidopsis cyt1* mutants are deficient in a mannose-1-phosphate guanylyltransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis. Proc Natl Acad Sci USA **98:** 2262–2267

Macknight R, Bancroft I, Page T, Lister C, Schmidt R, Love K, Westphal L, Murphy G, Sherson S, Cobbett C, et al (1997) *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell **89:** 737–745

Macknight R, Duroux M, Laurie R, Dijkwel P, Simpson G, Dean C (2002) Functional significance of the alternative transcript processing of the *Arabidopsis* floral promoter FCA. Plant Cell **14:** 877–888

Martinez C, Pons E, Prats G, Leon J (2004) Salicylic acid regulates flowering time and links defence responses and reproductive development. Plant J **37:** 209–217

Martinez-Zapater JM, Coupland G, Dean C, Koornneef M (1994) The transition to flowering in *Arabidopsis*. *In* EM Meyerowitz, CR Somerville, eds, Arabidopsis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 403–411

Michaels SD, Amasino RM (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11:** 949–956

Miller G, Suzuki N, Rizhsky L, Hegie A, Koussevitzky S, Mittler R (2007) Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. Plant Physiol **144:** 1777–1785

Mizoguchi T, Wright L, Fujiwara S, Cremer F, Lee K, Onouchi H, Mouradov A, Fowler S, Kamada H, Putterill J, et al (2005) Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in *Arabidopsis*. Plant Cell **17:** 2255–2270

Mockler T, Yang H, Yu X, Parikh D, Cheng YC, Dolan S, Lin C (2003) Regulation of photoperiodic flowering by Arabidopsis photoreceptors. Proc Natl Acad Sci USA **100:** 2140–2145

Mockler TC, Guo H, Yang H, Duong H, Lin C (1999) Antagonistic actions of *Arabidopsis* cryptochromes and phytochrome B in the regulation of floral induction. Development **126:** 2073–2082

Oda A, Fujiwara S, Kamada H, Coupland G, Mizoguchi T (2004) Antisense suppression of the *Arabidopsis PIF3* gene does not affect circadian rhythms but causes early flowering and increases *FT* expression. FEBS Lett **557:** 259–264

Olmos E, Kiddle G, Pellny T, Kumar S, Foyer C (2006) Modulation of plant morphology, root architecture, and cell structure by low vitamin C in *Arabidopsis thaliana*. J Exp Bot **57:** 1645–1655

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. Plant Cell **15:** 939–951

Pavet V, Olmos E, Kiddle G, Mowla S, Kumar S, Antoniw J, Alvarez ME, Foyer CH (2005) Ascorbic acid deficiency activates cell death and disease resistance responses in Arabidopsis. Plant Physiol **139:** 1291–1303

Pnueli L, Liang H, Rozenberg M, Mittler R (2003) Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (*Apx1*)-deficient *Arabidopsis* plants. Plant J **34:** 187–203

Putterill J, Laurie R, Macknight R (2004) It's time to flower: the genetic control of flowering time. Bioessays **26:** 363–373

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The *CONSTANS* gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell **80:** 847–857

Quesada V, Macknight R, Dean C, Simpson GG (2003) Autoregulation of *FCA* pre-mRNA processing controls *Arabidopsis* flowering time. EMBO J **22:** 3142–3152

Queval G, Issakidis-Bourguet E, Hoeberichts FA, Vandorpe M, Gakiere B, Vanacker H, Miginiac-Maslow M, Van Breusegem F, Noctor G (2007) Conditional oxidative stress responses in the Arabidopsis photorespiratory mutant *cat2* demonstrate that redox state is a key modulator of daylength-dependent gene expression, and define photoperiod as a crucial factor in the regulation of H_2O_2-induced cell death. Plant J **52:** 640–657

Reed JW, Nagpal P, Poole DS, Furuya M, Chory J (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell **5:** 147–157

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakurai N, Suzuki H, Hirai MY, Noji M, Saito K, et al (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J **44:** 653–668

Saunders DS (2005) Erwin Bunning and Tony Lees, two giants of chronobiology, and the problem of time measurement in insect photoperiodism. J Insect Physiol **51:** 599–608

Sawa M, Nusinow DA, Kay SA, Imaizumi T (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science **318:** 261–265

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre IA, Coupland G (1998) The *late elongated hypocotyl* mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93:** 1219–1229

Scholl R, Rivero-Lepinckas L, Crist D (1998) Growth of plants and preservation of seeds. *In* JM Martinez-Zapater, J Salinas, eds, Arabidopsis Protocols. Humana Press, Totowa, NJ, pp 3–5

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES (1999) The *FLF* MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. Plant Cell **11:** 445–458

Simon R, Igeno MI, Coupland G (1996) Activation of floral meristem identity genes in *Arabidopsis*. Nature **384:** 59–62

Smirnoff N (2000) Ascorbate biosynthesis and function in photoprotection. Philos Trans R Soc Lond B Biol Sci **355:** 1455–1464

Smirnoff N, Dowdle J, Ishikawa T (2007) The role of VTC2 in vitamin C biosynthesis in *Arabidopsis thaliana*. Comp Biochem Physiol A **146:** S250

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Mas P, Panda S,

Kreps JA, Kay SA (2000) Cloning of the Arabidopsis clock gene *TOC1*, an autoregulatory response regulator homolog. Science **289:** 768–771

Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. Nature **410:** 1116–1120

Tournois J (1912) Influence de la lumière sur la floraison du houblon japonais et du chanvre déterminées par des semis haitifs. C R Acad Sci Ser III Sci Vie **155:** 297–300

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science **303:** 1003–1006

Von Tiedemann A (1997) Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. Physiol Mol Plant Pathol **50:** 151–166

Wang ZY, Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell **93:** 1207–1217

Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature **393:** 365–369

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D (2005) Integration of spatial and temporal information during floral induction in *Arabidopsis*. Science **309:** 1056–1059

Wilson RN, Heckman JW, Somerville CR (1992) Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. Plant Physiol **100:** 403–408

Wolucka BA, Goossens A, Inze D (2005) Methyl jasmonate stimulates the *de novo* biosynthesis of vitamin C in plant cell suspensions. J Exp Bot **56:** 2527–2538

Wormuth D, Baier M, Kandlbinder A, Scheibe R, Hartung W, Dietz KJ (2006) Regulation of gene expression by photosynthetic signals triggered through modified CO_2 availability. BMC Plant Biol **6:** 15

Yang HQ, Wu YJ, Tang RH, Liu D, Liu Y, Cashmore AR (2000) The C termini of Arabidopsis cryptochromes mediate a constitutive light response. Cell **103:** 815–827

Yanovsky MJ, Kay SA (2003) Living by the calendar: how plants know when to flower. Nat Rev Mol Cell Biol **4:** 265–275