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EARLY FLOWERING3 Regulates Flowering in Spring Barley by Mediating Gibberellin Production and *FLOWERING LOCUS T* Expression[C][W]

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***EARLY FLOWERING3* (*ELF3*) is a circadian clock gene that contributes to photoperiod-dependent flowering in plants, with loss-of-function mutants in barley (*Hordeum vulgare*), legumes, and *Arabidopsis thaliana* flowering early under noninductive short-day (SD) photoperiods. The barley *elf3* mutant displays increased expression of *FLOWERING LOCUS T1* (*FT1*); however, it remains unclear whether this is the only factor responsible for the early flowering phenotype. We show that the early flowering and vegetative growth phenotypes of the barley *elf3* mutant are strongly dependent on gibberellin (GA) biosynthesis. Expression of the central GA biosynthesis gene, *GA20oxidase2*, and production of the bioactive GA, GA_1, were significantly increased in *elf3* leaves under SDs, relative to the wild type. Inhibition of GA biosynthesis suppressed the early flowering of *elf3* under SDs independently of *FT1* and was associated with altered expression of floral identity genes at the developing apex. GA is also required for normal flowering of spring barley under inductive photoperiods, with chemical and genetic attenuation of the GA biosynthesis and signaling pathways suppressing inflorescence development under long-day conditions. These findings illustrate that GA is an important floral promoting signal in barley and that *ELF3* suppresses flowering under noninductive photoperiods by blocking GA production and *FT1* expression.**

INTRODUCTION

The induction of flowering is a key developmental decision in a plant's life cycle, and its timing is an important adaptive trait for both wild and domesticated plants. The duration of light during the day, known as photoperiod, is one environmental signal used by plants to identify conditions favorable for flowering. Flowering in plants such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), pea (*Pisum sativum*), and *Arabidopsis thaliana* is strongly promoted under long-day (LD) conditions, with transcriptional activation of *FLOWERING LOCUS T*-like genes (*FT1* in barley) being a key determinant of the flowering response. During crop domestication, however, breeders have identified plants that display reduced photoperiod sensitivity to assist migration of crops to latitudes where the shorter daylengths would otherwise impede floral induction (Pugsley, 1983; Beales et al., 2007; Weller et al., 2012; Zakhrabekova et al., 2012). These modifications have also facilitated crop development in marginal environments that benefit from early flowering due to reduced water availability and increased temperatures at grain maturity (Gustafsson et al., 1971; Pugsley, 1983; Worland et al., 1998; Jones et al., 2008).

A major regulator of photoperiod sensitivity is the circadian clock, an endogenous mechanism used by organisms to establish a biological rhythm according to the 24-h day-night cycle (McClung, 2006). Recent studies have shown that mutations in circadian clock genes are responsible for the modified photoperiod sensitivity of numerous crop plants (Turner et al., 2005; Beales et al., 2007; Murphy et al., 2011; Faure et al., 2012; Matsubara et al., 2012; Weller et al., 2012; Zakhrabekova et al., 2012). *EARLY FLOWERING3* (*ELF3*), for example, is a component of the circadian clock that regulates photoperiod sensitivity in barley, pea, and *Arabidopsis*, as loss-of-function mutations of this gene promote rapid flowering under both short-day (SD) and LD conditions (Zagotta et al., 1996; Faure et al., 2012; Weller et al., 2012; Zakhrabekova et al., 2012). *ELF3* is an evening-expressed gene that encodes a nuclear-localized protein critical for gating the input of light signals to the circadian clock and regulating the expression of core clock oscillator genes (Covington et al., 2001; Hicks et al., 2001). During the nighttime, ELF3 represses the activity of core circadian clock genes as well as output genes that are regulated by the clock, and it is essential for maintaining correct diurnal expression patterns (Thines and Harmon, 2010; Dixon et al., 2011; Nusinow et al., 2011). The loss of ELF3 function facilitates photoperiod-insensitive early flowering and other developmental phenotypes, such as increased

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elongation of hypocotyls during vegetative growth (Zagotta et al., 1996; Nusinow et al., 2011).

Recently, *ELF3* was identified as the gene responsible for the early photoperiod-insensitive flowering of the barley mutant *praematurum.a-8* (*mat.a-8*), which is allelic to the *early maturity8* mutant (Faure et al., 2012; Zakhrabekova et al., 2012). Consistent with findings from *Arabidopsis*, barley loss-of-function *elf3* mutants display arrhythmic expression of circadian clock genes when plants are shifted from day-night cycles to constant light and defective repression of clock output genes during the nighttime (Faure et al., 2012; Zakhrabekova et al., 2012). Similarly, the expression of the key flowering gene, *FT1*, is derepressed in barley *elf3* mutant plants grown under SD conditions, displaying a strong peak of expression during the nighttime relative to plants with a functional *ELF3* gene (Faure et al., 2012; Hemming et al., 2012). While the increased transcription of *FT1* is consistent with the early flowering phenotype of *elf3*, the mechanism by which *ELF3* regulates *FT1* expression is not known, and it is unclear whether this is the only cause of the early flowering.

In this study, we investigated the early flowering phenotype of the *elf3* barley mutant, *mat.a-8*, in comparison to the progenitor cultivar 'Bonus' that contains a functional *ELF3* gene, which will henceforth be referred to as *elf3* and the wild type, respectively (Gustafsson et al., 1960, 1971; Zakhrabekova et al., 2012). We show that the early flowering and vegetative growth phenotypes of *elf3* plants are explained in part by increased production of the hormone gibberellin (GA). We also show that GA has an important role during the floral transition in barley by acting cooperatively with FT1 under inductive photoperiods to activate expression of floral identity genes at the developing inflorescence. Our results suggest that *ELF3* is required to maintain photoperiod sensitivity in spring barley by suppressing *FT1* expression and production of active GAs, when plants are grown under noninductive photoperiods.

RESULTS

The Vegetative and Early Flowering Phenotypes of the *elf3* Mutant Are GA Dependent

In preparation for our analysis of flowering time, wild-type and *elf3* plants were grown under SD conditions. During early vegetative growth, *elf3* plants displayed pleiotropic phenotypes, including elongated coleoptiles and long, pale-green leaves (Figure 1A). Quantitative measurements of these phenotypes showed that *elf3* plants had an accelerated rate of germination, decreased chlorophyll concentration, elongated coleoptiles, and increased leaf length and rate of leaf growth, relative to the wild type (Figures 1B to 1F). These phenotypes collectively resemble increased GA responses (Wolf and Haber, 1960; Huang et al., 1998; Hauvermale et al., 2012) and implied that *elf3* plants have either higher GA levels or constitutive GA responses. To investigate this hypothesis, *elf3* and wild-type plants were treated with the GA biosynthesis inhibitor paclobutrazol (PAC). Application of increasing amounts of PAC strongly suppressed the rate of leaf growth in *elf3* plants, reducing growth rates to wild-type levels (Figure 1F). As *elf3* plants exhibited increased sensitivity to PAC, we conclude that *elf3* plants do not have a constitutive GA response.

Similar to the growth phenotypes observed in *elf3* barley plants, *Arabidopsis elf3* mutants display elongated hypocotyls, due to an inability to correctly regulate diurnal expression of growth promoting transcription factors *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) and *PIF5* (Thines and Harmon, 2010; Nusinow et al., 2011). DELLA proteins repress the activity of PIF transcription factors, which is relieved by application of GA (de Lucas et al., 2008; Feng et al., 2008). Consequently, plants with increased levels of PIFs display increased sensitivity to GA and reduced sensitivity to PAC (de Lucas et al., 2008). To determine whether barley *elf3* plants display a similar increase in GA sensitivity, we measured the response of both genotypes to GA_3 application following treatment with PAC. By measuring the rate of leaf growth, we observed that *elf3* and wild-type plants respond similarly to low, moderate, and high concentrations of exogenous GA_3 (Figure 1G). Taken together, we conclude that *elf3* plants do not have increased GA sensitivity during vegetative phases of development and that the growth phenotypes of *elf3* plants are highly dependent on GA biosynthesis.

As GA regulates diverse aspects of plant growth and development, including the transition to flowering (Hauvermale et al., 2012), we investigated whether a GA-dependent pathway is involved in the photoperiod-insensitive early flowering phenotype of the *elf3* barley mutant. To this end, we analyzed the effect of PAC treatment on flowering in both wild-type and *elf3* plants grown under SD conditions (Figures 2A and 2B; Supplemental Figure 1). PAC treatment dramatically suppressed the early flowering phenotype of *elf3* plants, while control and PAC-treated wild-type plants did not flower during the course of the experiment (Figure 2B; Supplemental Figure 1). Dissection of developing spikes on the day of emergence for the control *elf3* plants showed that PAC treatment strongly suppressed inflorescence development, with increasing amounts of PAC progressively delaying the stage of spike maturity (Figure 2A; Supplemental Figure 1). PAC treatment also inhibited development of immature inflorescences from wild-type plants, with the strongest PAC concentration preventing progression beyond the double ridge stage (Supplemental Figure 1A). The suppressive effect of PAC treatment on *elf3* flowering could be restored by application of GA_3, confirming that reduced GA levels caused the delayed flowering (Figure 2; Supplemental Figure 1). In addition, GA application alone was able to slightly accelerate flowering in *elf3* plants but was not able to promote complete flowering in wild-type plants that were not expressing *FT1* (Figure 2; Supplemental Figure 1C). Taken together, these results suggest that a GA-dependent pathway contributes to the early flowering phenotype of the *elf3* plants.

GA Promotes Flowering in *elf3* Independently of Changes in *FT1* Expression

The transition to flowering is regulated by genes whose expression is triggered by exposure to inductive conditions. *FT*, for example, is an important regulator of flowering that is transcriptionally activated under inductive photoperiods (Kardailsky et al., 1999; Kobayashi et al., 1999). In barley, *FT1* is the main *FT*-like gene involved in the switch from vegetative to reproductive development and it is highly expressed under LD photoperiods, relative to SD (Yan et al., 2006; Faure et al., 2007). Previously, it was shown that *elf3* barley plants

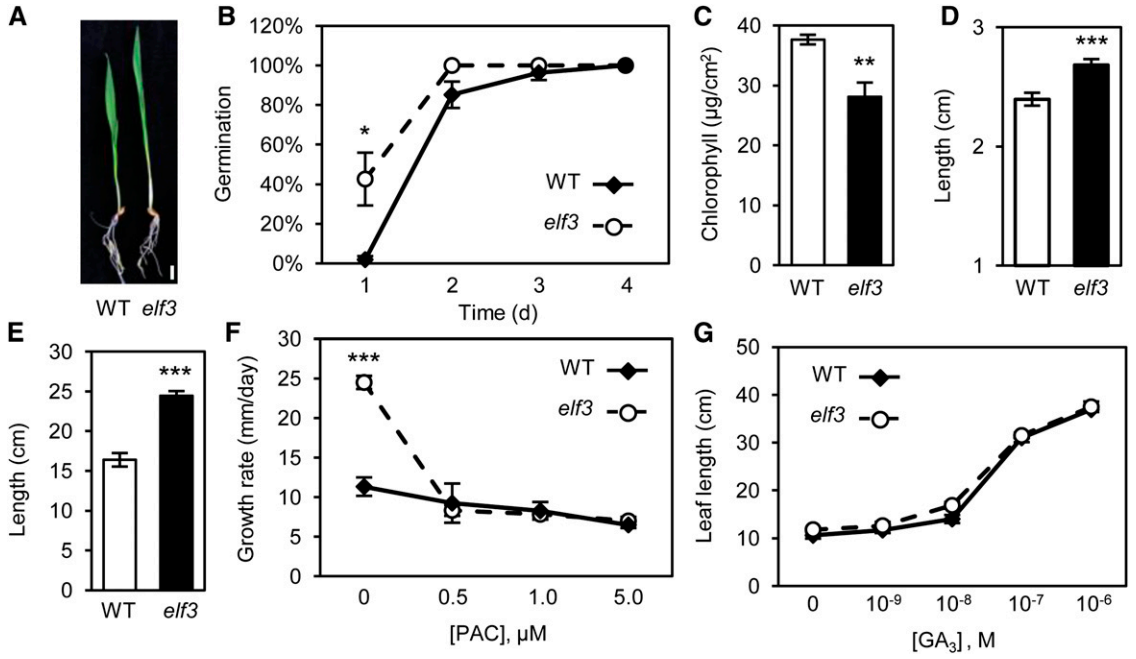

Figure 1. *elf3* Plants Exhibit Phenotypes Consistent with Increased GA Production.

(A) *elf3* plants display increased growth and pale green leaves compared with the wild type. Bar = 1 cm.
(B) to **(E)** Quantification of germination rate **(B)**, chlorophyll concentration **(C)**, coleoptile length **(D)**, and leaf length **(E)** in wild-type and *elf3* plants.
(F) Growth rate of *elf3* plants is dramatically reduced to wild-type levels by PAC treatment.
(G) Wild-type and *elf3* plants display identical sensitivity and response to GA application following PAC treatment (1 μM). Data are the mean ± SE of 10 biological replicates (*P < 0.05; **P < 0.01; ***P < 0.001). Plants were grown in SDs.

contain increased levels of *FT1* under noninductive photoperiods (Faure et al., 2012; Hemming et al., 2012) (Supplemental Figure 2), which likely explains, at least in part, the photoperiod-insensitive flowering of *elf3* plants. To determine if the inhibition effect of PAC on flowering is caused by reduced expression of *FT1*, we compared *FT1* transcript levels from leaves of PAC-treated and control plants grown under SD conditions. Analysis of *FT1* transcript levels in wild-type and *elf3* plants at each of the PAC concentrations used in this study, and diurnal expression analysis in PAC-treated and control *elf3* plants, revealed that PAC treatment did not significantly affect levels of *FT1* transcripts (*FT1* transcripts were not detected in wild-type plants) (Figures 2C and 2D). These results suggest that the delay in flowering caused by inhibiting GA biosynthesis was independent of changes in *FT1* expression and that the early flowering phenotype of *elf3* plants is not wholly explained by increased expression of *FT1*.

Given that gibberellins promote flowering in *Arabidopsis* through the expression of genes with important roles in the shoot apical meristem (Blázquez and Weigel, 2000; Moon et al., 2003; Yu et al., 2004; Eriksson et al., 2006; Jung et al., 2012), we compared the transcription of such genes within developing spikes of wild-type and *elf3* plants. We also compared the expression of these genes in developing spikes of control *elf3* plants to those that had been treated with PAC and PAC/GA to determine if their expression is GA dependent (Figure 3). The apex samples used for this analysis were harvested at the fourth-leaf stage when the *elf3* apices displayed the very initial signs of progressing beyond the transition apex stage and were therefore still developmentally comparable to the apices of wild-type plants and the PAC-treated *elf3* plants. The genes analyzed included *LEAFY* (*LFY1*), *SUPPRESSOR OF CONSTANS1* (*SOC1*), *FLORAL PROMOTING FACTOR1* (*FPF1*), *FPF2*, *FPF3*, and the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes *SPL11*, *SPL12*, and *SPL14* (Greenup et al., 2010; Papaefthimiou et al., 2012). We also examined the expression of genes with important roles during early inflorescence development in cereals, including *VERNALIZATION1* (*VRN1*), *MADS8*, *MADS14*, and *PANICLE PHYTOMER2* (*PAP2*) (Trevaskis et al., 2003; Yan et al., 2003; Kobayashi et al., 2010, 2012). From this analysis, we identified three gene categories: genes with increased expression in *elf3* compared with the wild type that respond to changes in GA levels (Figure 3A; GA dependent; *SOC1*, *FPF3*, *LFY1*, and *PAP2*), genes with increased expression in *elf3* compared with the wild type that do not respond to changes in GA levels (Figure 3B; *MADS8* and *MADS14*), and genes that are equally expressed in *elf3* and wild-type plants (Figure 3C; *ELF3* independent; *VRN1*, *SPL12*, and *SPL14*). We did not detect expression of *FPF1*, *FPF2*, or *SPL11* in any of the apex samples. These results suggest that GA is required to promote the early flowering phenotype of *elf3* plants by switching on expression of genes that are important for inflorescence development, including *SOC1*, *FPF3*, *LFY1*, and *PAP2*.

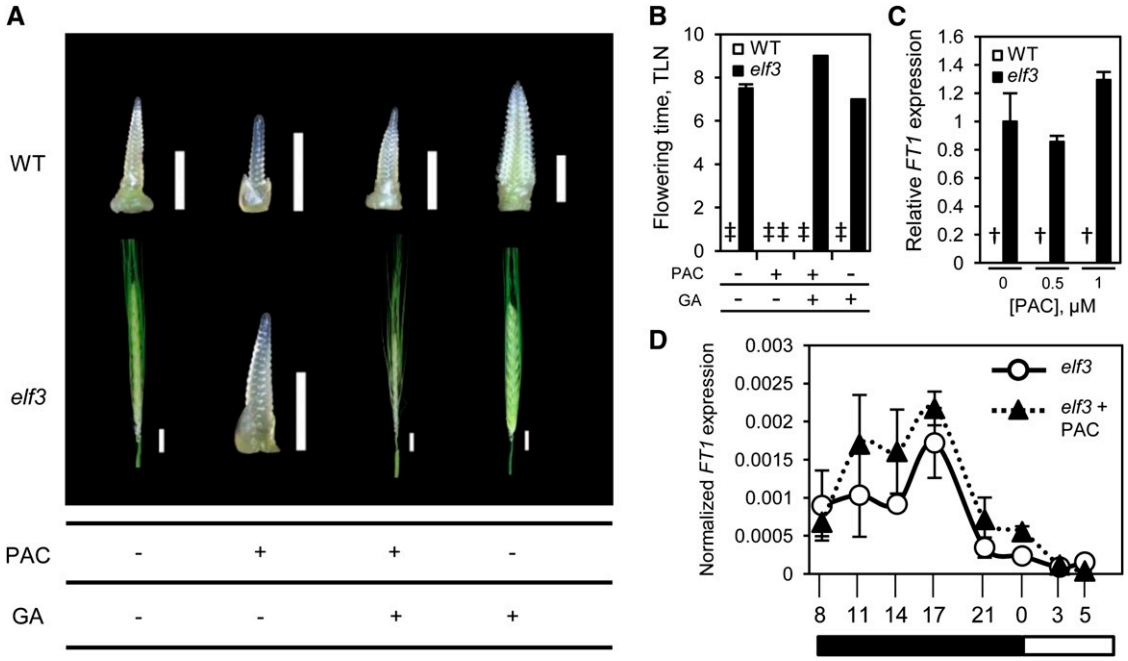

Figure 2. The Early Flowering Phenotype of *elf3* Plants Is GA Dependent.

Inflorescence development **(A)** and developmental flowering time **(B)** of wild-type and *elf3* plants under SD conditions following treatment with PAC (1 μM), PAC (1 μM) and GA_3 (10^{-8} M), or GA_3 (10^{-8} M). The images of inflorescences were taken on the day when the spike of the *elf3* control plant emerged from the boot. Bars for immature inflorescences = 1 mm and for mature spikes = 1 cm. Data are the mean ± SE of eight biological replicates. The delay in flowering by PAC treatment, at all concentrations tested **(C)** and throughout the 24-h period **(D)** (PAC; 1 μM), is not caused by reduced expression of *FT1*. Black and white rectangles illustrate periods of dark and light, respectively. Data are the mean ± SE of three biological replicates (†, no *FT1* transcripts detected). All data are from plants grown under SDs, and the RNA for *FT1* transcript analysis was harvested from plants at the fourth leaf stage. TLN, total leaf number; ‡, plants did not flower.

GA Production Increases in the Absence of ELF3

The vegetative and reproductive developmental phenotypes of *elf3* plants suggest they have increased GA responses that are sensitive to inhibition of GA biosynthesis. It is possible, therefore, that *elf3* plants produce higher levels of GA than the wild type. To test this hypothesis, we first measured the transcript levels of *GA20oxidase* (*GA20ox*) genes, which catalyze late stages of active-GA production and whose expression is light responsive (Xu et al., 1995; Huang et al., 1998; King et al., 2006). In addition to the three *GA20ox* genes that have been reported in barley (*GA20ox1*, *GA20ox2*, and *GA20ox3*) (Spielmeyer et al., 2004; Jia et al., 2009), we surveyed the barley genome for additional *GA20ox* genes, as five *GA20ox* genes are present in the *Arabidopsis* genome (Hedden et al., 2001). Based on amino acid sequence similarity and genetic relatedness to other *GA20ox* genes, we identified a fourth putative *GA20ox* gene, *GA20ox4* (MLOC_34543) (Supplemental Figures 3 and 4). Diurnal transcript analysis of *GA20ox2* revealed high expression during the nighttime in *elf3* plants relative to the wild type, where transcription appeared to be repressed (Figure 4A). *GA20ox1*, *GA20ox3*, and *GA20ox4* were also more highly expressed during the nighttime at ZT 21 h, although expression was detected at lower levels than for *GA20ox2* (Supplemental Figure 5). This result is consistent with a role for *ELF3* in repressing the expression of clock-output genes during the nighttime, which is supported by the pattern of *ELF3* expression in wild-type barley being strongest between ZT 14 h and 21 h (Figure 4B). Expression analysis of *GA20ox2* and *GA20ox3* after GA_3 application suggests that the increased expression of *GA20ox* in *elf3* plants is not due to a defective feedback mechanism, as transcript levels were significantly reduced in wild-type and *elf3* plants treated with GA_3, relative to control plants (Supplemental Figure 5).

We also measured the expression of *GA20ox* in the apex using the samples prepared for analysis of floral identity genes, as it was recently proposed that flowering is promoted in wheat via activation of *GA20ox* in the developing apex (Pearce et al., 2013). We did not detect expression of *GA20ox1* in the apex, and *GA20ox3* and *GA20ox4* were very weakly expressed, with no difference detected between genotypes or treatments (Supplemental Figure 5). While *GA20ox2* was expressed in the apex, it was detected at equal levels in *elf3* and wild-type plants (Figure 4C). In further support of the feedback response of GA biosynthesis genes functioning in the *elf3* plants, PAC treatment strongly increased *GA20ox2* expression in the apex, which was subsequently reduced by application of GA_3 (Figure 4C).

Taken together, these results suggest there is an increased production of GA in the leaves of *elf3* plants but not in the developing apex. To directly test this hypothesis, we measured the amount of bioactive GA_1 produced in leaves of *elf3* and wild-type plants, as well as levels of the precursor molecules, GA_{19}

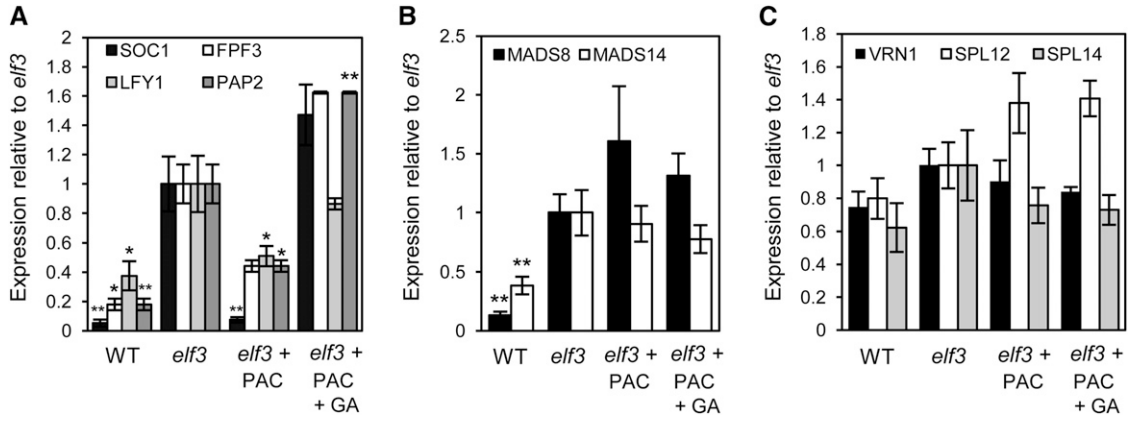

Figure 3. Expression Analysis of Floral Identity Genes in the Developing Apex.

Quantitative RT-PCR analysis of floral identity genes in developing inflorescences of wild-type and *elf3* plants identifies genes that are GA dependent **(A)**, more highly expressed in *elf3* than in the wild type but not responsive to changes in GA levels **(B)**, or equally expressed in wild-type and *elf3* plants (*ELF3* independent) **(C)**. PAC and GA_3 treatment concentrations were 1 μM and 10^{-7} M, respectively. Plants were grown in SD conditions, and apex samples were collected from plants at the fourth leaf stage. Data are the mean ± SE of three biological replicates, each containing six developing inflorescences (*$P < 0.05$; **$P < 0.01$).

and GA_{20}, and the inactivation product, GA_8. We found significantly higher levels of GA_1, GA_{19}, and GA_{20} in *elf3* leaves compared with the wild type (Figure 4D; Supplemental Table 1). These results are consistent with the increased expression of *GA20ox2* detected in *elf3* plants, as GA20ox catalyzes the production of GA_{19} and GA_{20}, with GA_{20} subsequently used for formation of GA_1 (Xu et al., 1995). We also detected increased levels of GA_8 in *elf3* plants, relative to the wild type (Figure 4D; Supplemental Table 1). GA_8 is a metabolite produced by catabolism of GA_1, and increased levels of GA_8 are typical of systems that contain elevated amounts of GA_1 (Davies and Rappaport, 1975).

We also examined the expression of *GA3ox* and *GA2ox* genes in these samples to determine if they may contribute to the increased production of GA_1 and GA_8, respectively. Two paralogs for each of *GA3ox* and *GA2ox* have been described in barley (Spielmeyer et al., 2004). We surveyed the barley genome for additional genes, and based on amino acid sequence identity and genetic relatedness, we identified three additional putative *GA2ox* genes: MLOC_71202, MLOC_72016, and MLOC_38462. We named MLOC_38462 as *GA2ox3* because it displays a strong phylogenetic relationship to *GA2ox3* from rice (*Oryza sativa*; Supplemental Figures 6 and 7). In the leaf samples, we were able to detect transcripts for *GA3ox1*, *GA3ox2*, *GA2ox3*, and *GA2ox4* but not the other *GA2ox* genes (Figure 4E). Quantitative RT-PCR showed a significant increase in transcripts of *GA3ox1* during the daytime in *elf3* plants relative to the wild type, although it was very weakly expressed relative to *GA3ox2* (Figure 4E). *GA2ox3* transcripts were significantly higher during the nighttime in *elf3* plants relative to the wild type, while no significant difference was detected for *GA3ox1*, *GA3ox2*, or *GA2ox4* (Figure 4E). The increased expression of *GA2ox3* is consistent with the feedback mechanism of the GA biosynthesis pathway, as the *elf3* leaves contain more bioactive GA_1 than the wild type. In the apex samples, we detected transcripts for *GA3ox2* and *GA2ox3*, but not for the other genes (Figure 4F). Transcript levels for *GA3ox2* were greater in wild-type plants relative to *elf3*, while there was no difference in *GA2ox3* between the two genotypes (Figure 4F). Both *GA3ox2* and *GA2ox3* responded to chemical treatments that affected GA levels (Figure 4F). Taken together, these results demonstrate that *ELF3* is required to maintain correct expression of GA biosynthesis genes, particularly *GA20ox2* in the leaves, and confirm that increased GA production contributes to the vegetative and reproductive phenotypes observed in *elf3* plants.

Flowering in Spring Barley Is GA Dependent

The above-mentioned results suggest that increased GA biosynthesis promotes the early photoperiod-insensitive flowering and vegetative growth phenotypes of *elf3* plants. As GA also promotes flowering in other plant species under inductive photoperiods (King and Evans, 2003), we hypothesized that GA is necessary for LD-induced flowering in wild-type spring barley. A limitation of testing this hypothesis in cv Bonus is that it contains an allele of *PHOTOPERIOD DEPENDENT1* (*PPD-H1*) that does not respond strongly to LDs (Hemming et al., 2012). We therefore investigated the role of GA in flowering using a genotype (CSIRO B07; see Methods) that contains functional alleles of both *ELF3* and *PPD-H1* and is therefore responsive to photoperiod. This genotype has an obligatory requirement for LD to flower, as it remained vegetative when grown in SD but flowered rapidly under LD conditions (Supplemental Figure 8). An inductive role for GA and FT1 was supported by the increased expression of *FT1*, *GA20ox2*, and *GA20ox3* in the leaves that occurred when CSIRO B7 plants were transferred from SD to LD (Figure 5A; Supplemental Figure 8). The rapid flowering phenotype of CSIRO B7 under LDs was delayed by treatment with PAC but rescued by exogenous GA_3 (Figure 5B). Dissection of inflorescences revealed that PAC treatment suppressed inflorescence development to a stage similar to that observed in SD-grown plants (Figure 5C). Combined, these results confirm that LDs induce GA biosynthesis

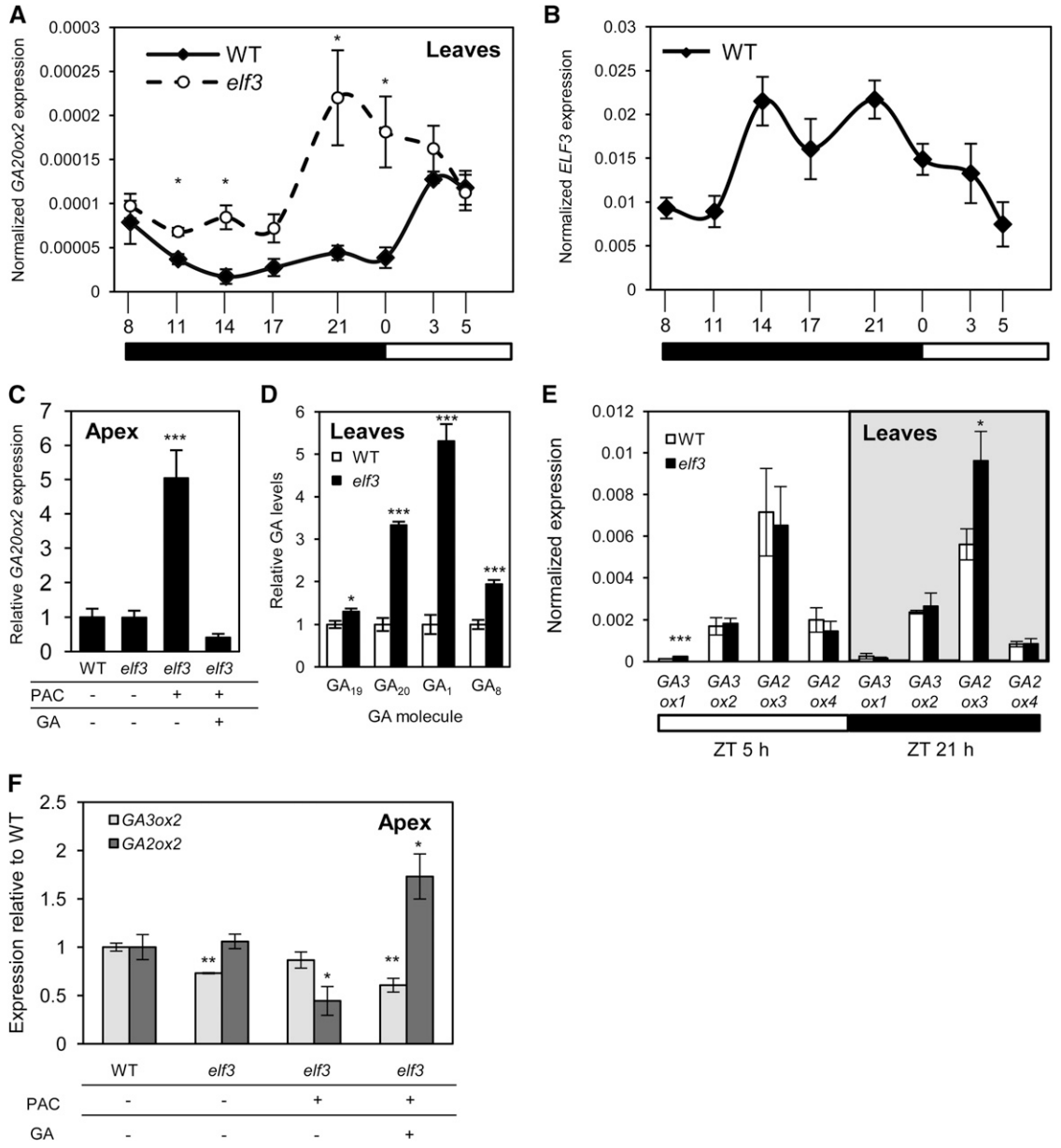

Figure 4. Expression of GA Biosynthesis Genes and Analysis of GA Levels in Wild-Type and *elf3* Plants.

(A) and **(B)** *GA20ox2* expression is elevated in *elf3* leaves during the nighttime phase of the diurnal cycle **(A)**, which overlaps **(B)** with the peak in expression of *ELF3* in wild-type plants. Numbers on the *x* axis refer to time (hour) within the 24-h cycle, with 0 h being dawn (lights on). Black and white rectangles illustrate periods of dark and light, respectively. Data are the mean ± SE of three biological replicates and are normalized to *GAPDH*.

(C) *GA20ox2* is expressed equally in the apex of wild-type and *elf3* plants but responds to exogenous application of PAC and GA. Data are the mean ± SE of three biological replicates, each containing six developing inflorescences. Values are relative to the wild type.

(D) Quantification of GA_{19}, GA_{20}, GA_1, and GA_8 levels in leaves of *elf3* plants relative to the wild type. Leaves were harvested from plants at the fourth leaf stage at ZT 0 h (dawn). Data are the mean ± SE of four biological replicates.

(E) Quantification of transcript levels for *GA3ox1*, *GA3ox2*, *GA2ox3*, and *GA2ox4* in leaves of wild-type and *elf3* plants during the day (ZT 5 h) and night (ZT 21 h). Black and white rectangles illustrate periods of dark and light, respectively. Data are the mean ± SE of three biological replicates.

(F) Quantification of *GA3ox2* and *GA2ox3* in the developing apices, as described in **(C)**. Data are the mean ± SE of three biological replicates, each containing six developing inflorescences. All data are from plants grown under SDs. (*P < 0.05; **P < 0.01; ***P < 0.001).

and *FT1* expression and that GA is required for flowering and spike development in spring barley.

Analysis of Flowering in GA Biosynthesis and Signaling Mutants

To further investigate an inductive role for GA in flowering of spring barley, we measured flowering time effects caused by mutations in GA biosynthesis and signaling genes. These included a loss-of-function mutant for the barley DELLA gene, *SLENDER1* (*SLN1*; *sln1c*), a partial loss-of-function *SLN1* mutant combined with the loss-of-function *SPINDLY1* (*SPY1*) mutation (*sln1s spy1a*), a gain-of-function *SLN1* mutant (*Sln1d*), a GID1 GA receptor loss-of-function mutant (*gse1a*), and a *GA3ox* biosynthesis mutant (*grd2c*) (Chandler and Robertson, 1999; Chandler et al., 2002; Chandler and Harding, 2013). All of these mutations are in the Himalaya genetic background, which is a photoperiod-responsive spring cultivar that contains functional *ELF3* and *PPD-H1* alleles. We hypothesized that lines with compromised GA production or signaling would flower later than Himalaya in LDs, while those with enhanced GA signaling should flower earlier. Consistent with this hypothesis, the *sln1c* and *sln1s spy1a* mutants flowered earlier than Himalaya, and the *gse1a*, *grd2c*, and *Sln1d* mutants flowered later (Figures 6A and 6B; Supplemental Figure 9). Inflorescence development was strongly impeded in the *gse1a*, *grd2c*, and *Sln1d* mutants (Figure 6C) and was restored in the *grd2c* mutant by application of GA_3 (Supplemental Figure 9), supporting a role for GA during floral induction and spike maturity. The compromised inflorescence development in these mutants often resulted in the failure of the spike from the main stem to emerge, such that it aborted during elongation of the flag leaf. The *sln1c* mutant produced a spike with infertile spikelets on the rachis, suggesting that a constitutively active GA pathway is also detrimental to inflorescence development (Figure 6C). We also measured LD expression of *FT1* in these mutants to determine whether the changes in flowering time were caused by altered transcriptional activity of *FT1*. None of the mutants displayed significantly different expression of *FT1* compared with Himalaya (Figure 6D), suggesting that genetic attenuation of the GA pathway does not affect flowering through changes in *FT1* activity, which is consistent with our analysis of *FT1* expression in PAC-treated *elf3* plants. Taken together, these results confirm that GA is an important signal that promotes flowering of spring barley independently of increased *FT1* transcription.

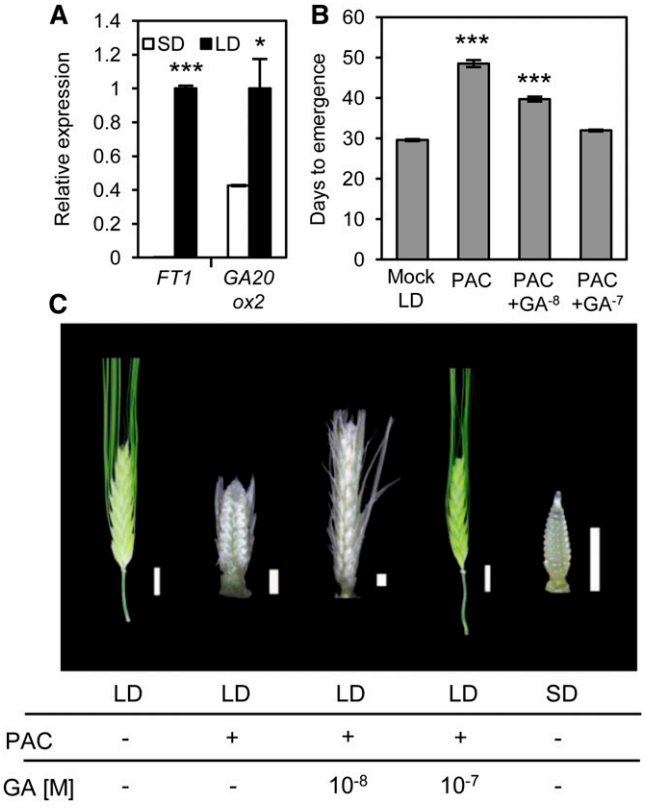

Figure 5. Flowering in Photoperiod-Responsive Spring Barley Is GA Dependent.

(A) *FT1* and *GA20ox2* expression in photoperiod-responsive spring barley increases under LD conditions. These data are from RNA extracted from leaf samples at the fourth leaf stage, harvested at ZT 16 h. Data are the mean ± SE of three biological replicates (*P < 0.05; ***P < 0.001).

(B) Flowering time in spring barley is delayed by PAC treatment and restored by application of GA_3. Data are the mean ± SE of 14 biological replicates (***P < 0.001).

(C) Inflorescence development of spring barley under LD conditions is GA dependent. The images of inflorescences were taken on the day when the spike of the LD control plant emerged from the boot. PAC concentration was 1 μM. Bars = 1 mm for immature inflorescences and 1 cm for mature spikes (green).

DISCUSSION

Variation in flowering time is a trait that is frequently used by breeders to improve yield performance of important crops in marginal growing environments. *ELF3* has been used to modify flowering time for the cultivation of barley and legumes in diverse growing regions, with loss-of-function mutants promoting photoperiod-insensitive early flowering (Faure et al., 2012; Weller et al., 2012; Zakhrabekova et al., 2012). Previous research, consistent with the results presented here, indicates that the early flowering phenotype of the barley *elf3* mutant is partially mediated by increased transcription of *FT1* (Faure et al., 2012; Hemming et al., 2012). In this article, we show that the early flowering phenotype is also dependent on increased GA biosynthesis, which is additionally responsible for the vegetative growth phenotypes of *elf3* plants. The floral promoting ability of GA is conserved in spring barley with a functional *ELF3* gene, and our results suggest that GA is an essential factor that acts cooperatively with *FT1* to promote flowering in this cereal crop.

ELF3 Regulates GA Production

Our results demonstrate that *ELF3* regulates GA production in barley by gating the transcriptional activity of genes that code for GA biosynthesis enzymes, in particular *GA20ox2*. As *ELF3* is a key component of the circadian clock, our results suggest that an important role of the clock in barley is to regulate production of GA. While this is a previously undescribed role for *ELF3*, it is consistent with the

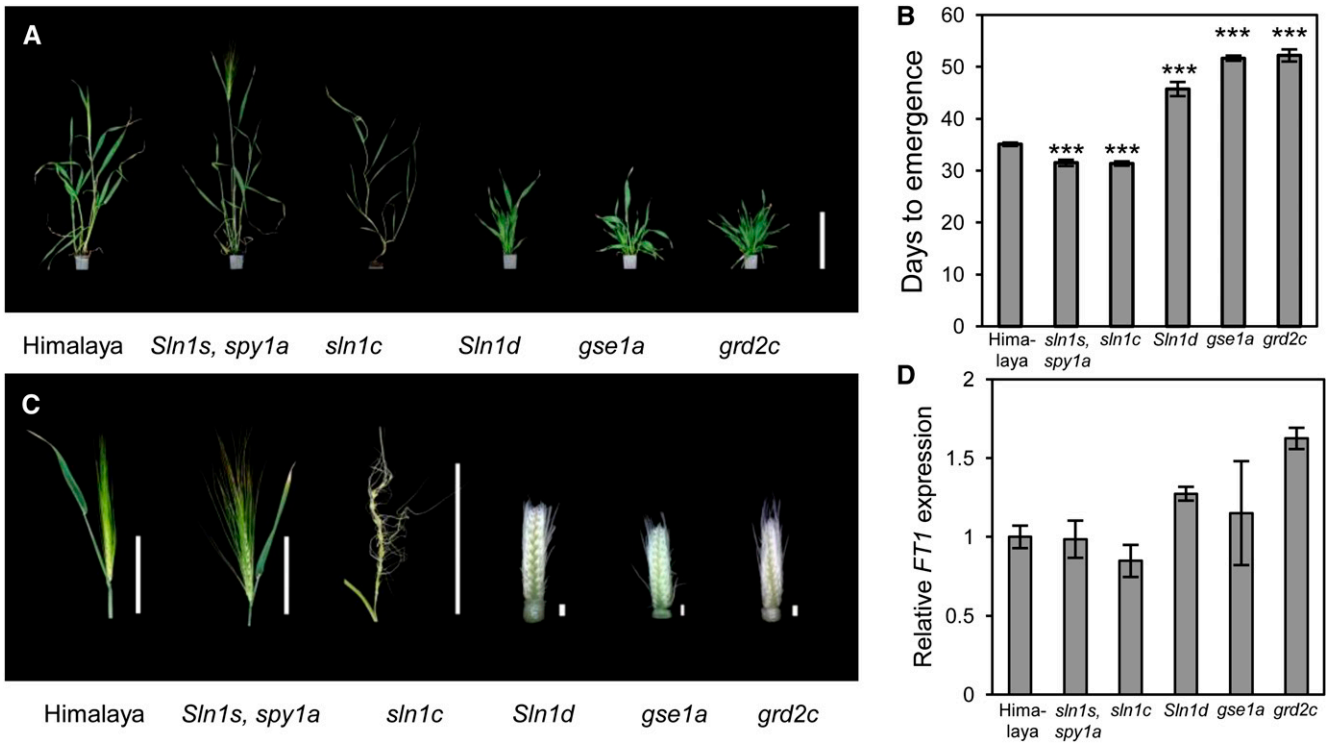

Figure 6. Flowering Time and Inflorescence Development Phenotypes of GA Biosynthesis and Signaling Mutants.

(A) and **(B)** Flowering time phenotypes of wild-type (Himalaya) and mutant plants with constitutive GA responses (*Sln1s*, *spy1a*, and *sln1c*) or compromised GA responses (*Sln1d*, *gse1a*, and *grd2c*) grown under LD conditions. Data for mutants with compromised GA responses include measurements of flowering of the first tiller in instances where the main stem failed to complete inflorescence development. Images were taken on the day of emergence for the wild-type (Himalaya) plants. Data are the mean ± SE of 10 biological replicates (***$P < 0.001$). Bars = 20 cm.

(C) Inflorescence development of wild-type (Himalaya) and GA biosynthesis and signaling mutants. The images of inflorescences were taken on the day when the spike of the LD grown Himalaya (wild-type) plant emerged from the boot. Bars = 1 mm for immature inflorescences and 10 cm for mature green spikes. The *sln1c* inflorescence is enlarged slightly to improve visibility.

(D) Relative *FT1* expression in leaves of wild-type (Himalaya) and GA biosynthesis and signaling mutants under LD conditions at ZT 16 h. Data are the mean ± SE of three biological replicates.

increased expression of *GA20ox* that occurs in *Arabidopsis* circadian clock mutants (Blázquez et al., 2002). The increased expression of *GA20ox* during the dark phase of the diurnal cycle is consistent with ELF3 functioning as a key repressor within the evening loop of the circadian clock (Fowler et al., 1999; Dixon et al., 2011). Based on results from model plants, it is possible that the increased expression of *GA20ox2* occurs through a process that directly involves ELF3; alternatively, it is a consequence of the *elf3* plants having a defective circadian clock. In *Arabidopsis*, ELF3 interacts with ELF4 and LUX ARRHYTHMO (LUX) to form an evening complex that directly represses transcription of clock output genes including *PIF4* and *PIF5* (Nusinow et al., 2011; Herrero et al., 2012). Loss of *ELF3* provokes increased expression of *PIF4* and *PIF5* during the dark, comparable to the loss of nighttime repression for *GA20ox* that we observed in *elf3* barley plants (Thines and Harmon, 2010; Nusinow et al., 2011). The possibility of a similar evening complex existing in barley is supported by the recent identification of *LUX1* as the candidate gene for the early maturity mutant, *eam10*, which displays an early-flowering phenotype and an increased rate of stem elongation (Campoli et al., 2013). Thus, it is possible that an ELF3/ELF4/LUX1 complex suppresses *GA20ox* transcription during the dark period of the diurnal cycle. ELF3 may also suppress *GA20ox* transcription via interaction with CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1). ELF3 interacts with COP1 to modulate light input signals to the circadian clock by destabilizing the GIGANTEA (GI) protein (Yu et al., 2008). In the absence of ELF3, GI protein levels are elevated during the dark period of a SD, which promotes increased transcription of floral promoting genes (Yu et al., 2008). As transcription of *GA20ox* increases under inductive photoperiods, it is plausible that ELF3 represses transcriptional activity of *GA20ox* during the dark period of SD via interaction with COP1. An alternate explanation for the increased levels of *GA20ox2* transcripts is that the loss of ELF3 function provokes elevated expression of *GA20ox2* via the absence of a correctly functional circadian clock, as observed by the irregular expression of the core oscillator genes *TOC1* and *CCA1* in this mutant (Faure et al., 2012; Zakhrabekova et al., 2012). For example, *PIF4* in *Arabidopsis* is more highly expressed in LD compared with SD (Lee and Thomashow, 2012) and is also significantly upregulated in *toc1* mutants compared with the wild type (Niwa et al., 2009). Given that *TOC1* expression is reduced during the nighttime in *elf3*

barley mutants relative to the wild type (Faure et al., 2012), it is possible that the increased expression of *GA20ox2* in the *elf3* mutant and in LD compared with SD is mediated via a clock-dependent mechanism that involves *TOC1*.

The transcriptional upregulation of *GA20ox* and *FT1* observed in *elf3* plants and upon transition from SD to LD suggests a possible role for ELF3 in identifying when daylength is sufficient for LD plants to flower. ELF3 functions as a light zeitnehmer (time-taker) that gates the input of light signals to the circadian clock, facilitating accurate measurement of daylength (Hicks et al., 1996; McWatters et al., 2000). In the absence of *ELF3*, the circadian clock is arrested to a constitutive day (lights on) state (McWatters et al., 2000; Thines and Harmon, 2010), which is consistent with the photoperiod-insensitive early flowering of *elf3* plants under SDs. The gating of light signals by ELF3 is particularly important at ZT 16 h (McWatters et al., 2000; Thines and Harmon, 2010), which corresponds with the period of the diurnal cycle when *GA20ox2* and *FT1* levels were dramatically higher in SD-grown *elf3* compared with the wild type and also when these genes are upregulated in LD relative to SD. A role for ELF3 in regulating *FT1* and *GA20ox2* under SD is also consistent with our detection of increased transcripts for these genes in *elf3* plants at times when *ELF3* expression peaks in wild-type plants (ZT 14-21 h) (Figure 4; Supplemental Figure 2). Thus, *ELF3* may be important for suppressing expression of floral promoting genes in LD plants when grown under SD conditions (Figure 7), which is consistent with the precocious flowering of *Arabidopsis*, pea, and barley *elf3* mutants that occurs under SD conditions (Zagotta et al., 1992, 1996; Faure et al., 2012; Zakhrabekova et al., 2012; Weller et al., 2012). Our analysis suggests that *GA20ox2* is one of the floral promoting genes that are regulated by a pathway involving ELF3, and this is consistent with the association of *GA20ox2* to the *Sdw1/DENSO* locus that contributes to earliness of head emergence in barley (Jia et al., 2009; Comadran et al., 2012).

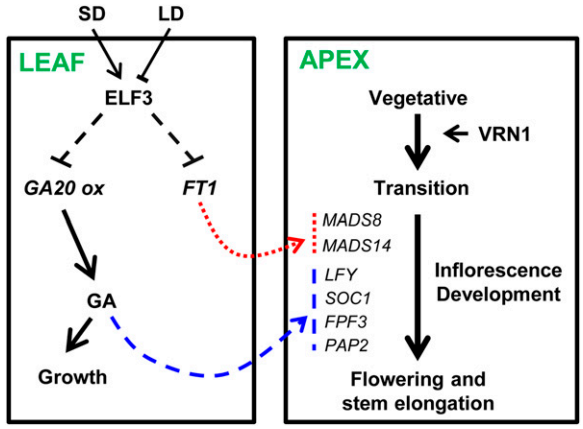

Figure 7. Model of ELF3 Regulation of Flowering in Spring Barley.

In SD-grown plants, ELF3 represses expression of *GA20ox* and *FT1* in leaves. GA20ox catalyzes rate-limiting steps in the production of bioactive GAs, which are able to promote vegetative growth and expression of floral identity genes (dashed arrow) at the developing apex (transition stage). *FT1* also promotes expression of floral identity genes (dotted arrow) in the developing apex. *VRN1* expression promotes the development of the vegetative apex to a stage that is competent to receive the promoting effect of GA and *FT1*, which are both required for the completion of inflorescence development and flowering.
[See online article for color version of this figure.]

GA Acts Cooperatively with *FT1* to Promote Flowering

Through attenuation of endogenous GA biosynthesis and signaling pathways, we were able to partly suppress the early flowering phenotype of *elf3* plants and delay floral development in spring barley. Taken together, these results suggest an essential role for GA in barley flowering. Interestingly, we observed that the GA-related suppression of flowering was independent of changes in *FT1* expression, which is consistent with studies in *Lolium* but contrary to those from *Arabidopsis* (King et al., 2006; Hisamatsu and King, 2008; Porri et al., 2012), suggesting there is variation among plants for the mechanism by which GA promotes flowering. The ability of exogenous GA_3 to rapidly restore flowering in PAC-treated plants only when *FT1* was present, and not when it was absent, suggests that GA and FT1 act coordinately to promote floral development in barley. Importantly, the completion of spike maturity that we observed when GA and FT1 are both present is distinct from the partial floral development that occurs in barley (Supplemental Figure 1), *Lolium*, and wheat when GA is applied under SD conditions and *FT* expression is absent (Macmillan et al., 2005; King et al., 2006; Pearce et al., 2013). We therefore propose that GA can promote flowering in barley but that it requires FT1 to complete inflorescence development (Figure 7). This model is in agreement with the original description of GA as a florigen, which proposed gibberellin is not the sole regulator of flower formation but that it participates in conjunction with other factors that are present under inductive photoperiods (Lang, 1957; Chailakhyan, 1958; Bernier et al., 1993). The bicomponent nature of the florigen signal (Chailakhyan, 1958) is also consistent with the idea that GA acts together with an anthesin (FT protein) to promote flowering (Chailakhyan, 1958). The dual requirement for *FT1* and GA may explain why only a low number of drastically early flowering mutants were identified in the large screen that produced *mat.a-8* (Gustafsson et al., 1960), as the photoperiod-insensitive Bonus cultivar would need to obtain a mutation that would simultaneously induce *FT1* and activate GA production to promote early flowering.

Based on our analysis of genes that are differentially expressed in the developing inflorescence of wild-type and *elf3* plants and their response to changes in GA levels, we propose that GA acts with *FT1* to promote flowering by activating expression of floral identity genes at the inflorescence meristem. In agreement with studies from *Arabidopsis* and wheat, we have shown that GA increases expression of *SOC1*, *FPF3*, *LFY1*, and *PAP2* in the developing inflorescence (Blázquez and Weigel, 2000; Moon et al., 2003; Achard et al., 2004; Eriksson et al., 2006; Pearce et al., 2013). We also identified flowering genes that do not respond to changes in GA levels, which are possibly *FT* dependent, as well as flowering genes that are equally expressed in the apices of *elf3* and wild-type plants that are independent of *ELF3*. One of the latter genes is *VRN1*, which promotes flowering in response to vernalization (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). Importantly, both the wild-type progenitor (Bonus) and CSIRO B07 contain spring alleles of *VRN1* that are expressed without exposure to prolonged cold. Our results are therefore consistent with findings from *Lolium* and wheat, whereby

exogenous GA is able to promote flowering only in winter plants that have been vernalized or spring plants that contain a constitutively highly expressed *VRN1* allele (Macmillan et al., 2005; Pearce et al., 2013). Consequently, we propose that *VRN1* expression in the apex is essential for development of a meristem that is competent to receive the floral inductive signals GA and FT1, which then promote flowering by activating transcription of a complete set of floral identity genes (Figure 7). This model is supported by PAC treatment not arresting meristem development at the vegetative stage, but allowing it to progress to the stage of transition apex, a step that requires *VRN1* and occurs immediately before the apex obtains a reproductive state (Waddington et al., 1983; Trevaskis et al., 2006). It is also consistent with *elf3* barley mutants that contain a winter allele of *VRN1* requiring vernalization to promote floral development, despite *FT1* (and probably also *GA20ox*) being expressed at increased levels (Faure et al., 2012).

Recently, Pearce et al. (2013) showed in wheat that LDs induce the expression of *FT* in leaves and *GA20ox* in the apex. Their model proposes that FT protein moves from the leaves to the apex where it induces expression of *GA20ox* and *VRN1*, which are required for normal spike development. Our results suggest that similar components promote flowering in spring barley but the mechanism of action is different. We found that LDs induce *FT1* and *GA20ox* in leaves and that both are essential for inflorescence differentiation and spike development. Based on our results, we propose that under noninductive SD conditions, ELF3 suppresses expression of *FT1* and *GA20ox* in leaves (Figure 7). In LDs, FT1 and GAs accumulate in the leaves and activate transcription of floral identity genes at the developing apex, presumably via translocation from the leaves to the apex. Importantly, we propose that both FT1 and GA are necessary for activation of all the floral identity genes required for completion of inflorescence development. The constitutively expressed *VRN1* allele present in spring barley is essential for the development of a competent meristem that can receive and respond to the floral inductive signals GA and FT1. While we cannot exclude the possibility that FT1 promotes expression of *GA20ox* in leaves, expression analysis in *Lolium* has shown that *GA20ox* transcripts accumulate more rapidly than *FT* upon transition from SD to LD (King et al., 2006). In addition, barley plants with functional alleles of *PPD-H1* that express *FT* at significantly higher levels than those with the insensitive *ppd-H1* allele do not exhibit vegetative phenotypes suggestive of increased GA activities (Turner et al., 2005). We therefore propose that GA and FT1 are simultaneously but independently produced in leaves under inductive LDs.

In conclusion, we demonstrated that the vegetative and reproductive phenotypes of *elf3* barley plants are promoted by excess production of GA. The early flowering phenotype of this mutant is dependent on GA even when *FT1* is expressed at high levels, and our evidence suggests that the requirement for both of these factors extends to spring barley for LD induction of flowering. The discovery that *ELF3* regulates GA content and *FT1* expression highlights a new role for this circadian clock gene, which may help breeding programs that are seeking to modify flowering time of cereals and extend the duration of spike development for improvement of crop yields.

METHODS

Plant Materials and Growth Conditions

Plant materials of spring barley (*Hordeum vulgare*) used in this study included Bonus (wild-type progenitor parent of *elf3*; *VRN1-1*, Δ*VRN2*, *ppd-H1*), the *elf3* mutant (*mat.a-8*), CSIRO B07 (*VRN1-7*, Δ*VRN2*, *PPD-H1*), and Himalaya (*VRN1-1*, Δ*VRN2*, *PPD-H1*). The GA pathway mutants of the Himalaya background included *sln1s/spy1a* (M251), *sln1c* (M770), *Sln1d* (M640), *gse1a* (M488), and *grd2c* (M489), as described previously (Chandler and Robertson, 1999; Chandler et al., 2002; Chandler and Harding, 2013). All plants were grown in Conviron CMP6050 growth cabinets at 20°C or in standard growth rooms at 23°C, both at 330 to 350 $\mu mol^{-2}\ s^{-1}$ PPFD. Plants were grown under one of two photoperiod regimes: SD (8 h light/16 h dark) or LD (16 h light/8 h dark).

Germination Assay

Germination assays were conducted in continuous dark on threshed seed that were after-ripened at 37°C for 4 weeks, as described previously (Gubler et al., 2008).

Chlorophyll Extraction and Measurements

Chlorophyll was extracted from fresh leaves (fifth-leaf stage) in acetone (100%) and measured spectrophotometrically at 645 and 663 nm (Arnon, 1949). Chlorophyll concentration was calculated using the formula: $(20.2 \times A_{645} + 8.02 \times A_{663})/cm^2$.

GA and PAC Treatments

All GA and PAC treatments were applied to plants grown on New Growool Propagating Blocks (Growool Horticultural Systems). GA treatments were performed using GA_3 (Sigma-Aldrich) prepared as a stock solution (10^{-2} M) in 95% ethanol and diluted in water prior to application to concentrations of 10^{-9} M, 10^{-8} M, 10^{-7} M, or 10^{-6} M, as indicated in the figures. PAC treatments were performed using PAC (Duchefe Biochemie) prepared as a stock solution (0.1 M) in dimethyl sulfoxide (Sigma-Aldrich) and diluted in water prior to application to concentrations of 0.5, 1, or 5 μM, as indicated in the figures. GA and PAC were applied to the plants by adding 500 mL solutions to the Growool for absorption via the roots. Treatments were applied twice per week until completion of the experiment.

Apex Dissection and Developmental Flowering Time Measurements

Apices were isolated with a binocular dissecting microscope and then digitally photographed on a Zeiss AxioCam MRc 5. Leaves were numbered sequentially from germination, and plants were grown until the flag leaf emerged to determine total leaf number. Heading date was measured as the day when the head first emerged from the sheath on the main stem (Zadoks scale, $Z = 47$).

RNA Extraction and Expression Analysis

RNA was extracted from the following plant material: (1) leaves from PAC-treated and control wild-type and *elf3* plants grown under SD, harvested at ZT 12 h (Figure 2C); (2) leaves from PAC-treated (1 μM) *elf3* plants grown under SD at defined intervals (Figure 2D); (3) apices from control, PAC-, and PAC/GA-treated wild-type and *elf3* plants grown under SD until the four-leaf stage (Figure 3); (4) leaves from wild-type and *elf3* plants grown under SD conditions harvested at defined intervals (Figure 4A); (5) leaves from CSIRO B07, Himalaya, and GA pathway mutants grown under SD or LD conditions at ZT 16 h (Figures 5A and 6D). Each leaf sample contained the youngest emerged leaf of two plants and was harvested from plants at the developmental fourth leaf stage. Each apex RNA

sample contained six-pooled apices harvested from the main stem. Transcript analysis was performed on total RNA extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich). Total RNA was treated with RQ1 DNase I (Promega) and reverse-transcribed with SuperScript III reverse transcriptase (Life Technologies), as per manufacturer's instructions. Quantitative RT-PCR was performed in a 7900HT Fast Real-time PCR system (Applied Biosystems) using SYBR green and Platinum Taq DNA polymerase (Life Technologies). All quantitative RT-PCR data points are the average of three biological replicates, with two technical replicates performed in each reaction. Expression of candidate genes was normalized against ACTIN and GAPDH. See Supplemental Table 2 for oligonucleotide sequences used for quantitative RT-PCR.

Measurement of GA Levels

GA measurements were performed on leaf and stem material collected from SD-grown wild-type and *elf3* plants at the four-leaf stage, with samples collected at ZT 0 h (lights on). Four replicates were performed for each genotype. Details of methods used for GA extraction and quantification are provided in Supplemental Methods.

Sequence Alignment and Phylogenetic Analysis

Barley GA20oxidase and GA2oxidase sequences were identified by BLAST search from public databases using the known GA20ox and GA2ox protein sequences from barley and *Arabidopsis thaliana* as bait (Supplemental Table 3). Multiple sequence alignments (Supplemental Data Sets 1 and 2) and construction of phylogenetic trees were performed as described previously (Boden et al., 2013). Bootstrap values are based on 1000 replicates for testing the significance of the nodes.

Statistical Analysis

Differences between treatments were tested by Student's *t* test. Results in figures are shown as means ± SE.

Accession Numbers

Sequence data from this article and their sources are provided in Supplemental Tables 2 and 3.

Supplemental Data

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

Supplemental Figure 1. Inhibition of GA Biosynthesis by PAC Treatment Delays Flowering and Inflorescence Development of *elf3* Plants.

Supplemental Figure 2. *FT1* Expression Is Elevated in the *elf3* Mutant, *mat.a-8*.

Supplemental Figure 3. Alignments of GA20oxidase Amino Acid Sequences.

Supplemental Figure 4. Maximum Likelihood Phylogenetic Tree of GA20oxidases.

Supplemental Figure 5. *GA20oxidase* Expression Analysis.

Supplemental Figure 6. Alignments of GA2oxidase Amino Acid Sequences.

Supplemental Figure 7. Maximum Likelihood Phylogenetic Tree of GA2oxidases.

Supplemental Figure 8. Flowering Time and Induction of *GA20ox3* in Photoperiod-Responsive Spring Barley Grown under SD and LD Photoperiods.

Supplemental Figure 9. Flowering Time of GA Biosynthesis and Signaling Mutants.

Supplemental Table 1. Measurements of GA_{19}, GA_{20}, GA_1, and GA_8 Levels from Wild-Type and *elf3* Plants.

Supplemental Table 2. Oligonucleotide Sequences Used in qRT-PCR Assays.

Supplemental Table 3. Gene Identifiers of the *GA20ox* and *GA2ox* Genes.

Supplemental Methods. GA Extraction and Quantification.

Supplemental Data Set 1. Text File of the Alignment of GA20oxidases Used for the Phylogenetic Analysis Shown in Supplemental Figure 4.

Supplemental Data Set 2. Text File of the Alignment of GA2oxidases Used for the Phylogenetic Analysis Shown in Supplemental Figure 7.

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AUTHOR CONTRIBUTIONS

S.A.B., D.W., and S.M.S. designed the research. S.A.B., D.W., J.J.R., N.W.D., and B.T. performed the research. B.T. and P.C. contributed new tools. S.A.B., D.W., J.J.R., N.W.D., and S.M.S. analyzed data. S.A.B., D.W., J.J.R., and S.M.S. wrote the article.

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