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Genetic and molecular dissection of the Drought Escape response in *A. thaliana*

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PART I

Abstract

Modulation of the transition to flowering plays an important role in the adaptation to drought. The drought escape (DE) response allows plants to adaptively shorten their life cycle to make seeds before severe stress leads to death. However, the molecular basis of DE response is unknown. The screen of different *Arabidopsis thaliana* flowering time mutants under DE– triggering conditions revealed the central role of the flower–promoting gene *GIGANTEA* (*GI*) and the florigen genes *FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*) in the DE response. Further screens showed that the phytohormone abscisic acid is required for DE response, positively regulating flowering under long day conditions (LDs). Drought stress promotes the transcriptional upregulation of the florigens in an ABA– and photoperiod– dependent manner, so that early flowering only occurs under LDs. Along with the florigens, the floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) is also up-regulated in a similar fashion and contributes to the activation of *TSF*. The DE response was recovered under short days in the absence of the floral repressor *SHORT VEGETATIVE PHASE* (*SVP*) or in *GI* overexpressing plants. Our data reveal a key role for GI in connecting photoperiodic cues and environmental stress independently from the central *FT*/*TSF* activator *CONSTANS*. This mechanism explains how environmental cues may act upon the florigen genes in a photoperiodically–controlled manner, thus enabling plastic flowering responses.

Introduction

1 Life cycle of Arabidopsis thaliana

Arabidopsis thaliana is a small weedy flowering plant, member of the *Brassicaceae* family and widely used as model organism in plant research. The life cycle of a wild type Arabidopsis is 4–6 weeks long and can be divided into three phases: germination, vegetative and reproductive (Fig. 1). Each phase transition is controlled according to internal and external cues. During germination the embryo starts its postembryonic life and develop a seedling that grow into an early (juvenile) vegetative phase. The vegetative phase change encompasses the gradual transition from juvenile to adult growth, when the plant becomes competent to undergo a floral transition. This marks the reproductive growth of the plant when flowers, fruits and seeds are produced (Bäurle and Dean, 2006).

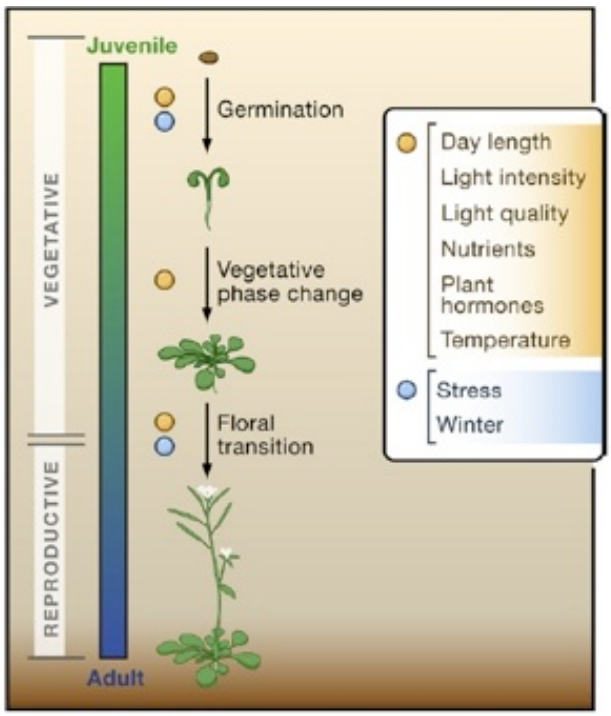

Figure 1. Developmental transition during the plant life cycle

All developmental transitions are regulated by environmental signals such as available nutrients, day length, light intensity, light quality, and ambient temperature as well as endogenous signals transmitted by plant hormones. Cold temperature and stress affect both germination and floral transition (Modified – B**ä**urle et al. 2006).

2 The regulation of the floral transition

The floral transition occurs when the shoot apical meristem (SAM) receives appropriate signals and switches from a vegetative to an inflorescence meristem. Proper timing of the floral transition is key to ensure reproductive success. For that to occur, plants perceive and integrate external and internal cues to start the reproductive growth in the most favourable conditions. Physiological, genetic and molecular analyses have led to the identification of four main pathways underlying the floral transition: the photoperiod, the vernalization, the autonomous and the gibberellin pathway.

2.1 The photoperiodic pathway

Arabidopsis is a facultative long-day plant. Flowering is promoted upon exposure to long days (LDs) and repressed under short days (SDs). The photoperiodic pathway is responsible for the discrimination between LDs and SDs conditions (Rédei, 1962; Koornneef et al., 1991). Three classes of photoreceptors, namely the PHYTOCHROMES (PHYs, red/far-red light receptor), the CRYPTOCHROMES (CRYs, blue/UV-A light receptor) and the FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) family (blue light receptor), detect different aspects of light such as intensity and quality (wavelength) (Thomas, 2006). The light input regulates at the transcriptional and posttranslational levels three key genes, that are required for correct day length measurement: *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) (Putterill et al., 1995; Fowler et al., 1999; Kobayashi et al., 1999). Mutations in any of these

genes result in a late flowering phenotype under LDs but have no, or only slight, effect under SDs.

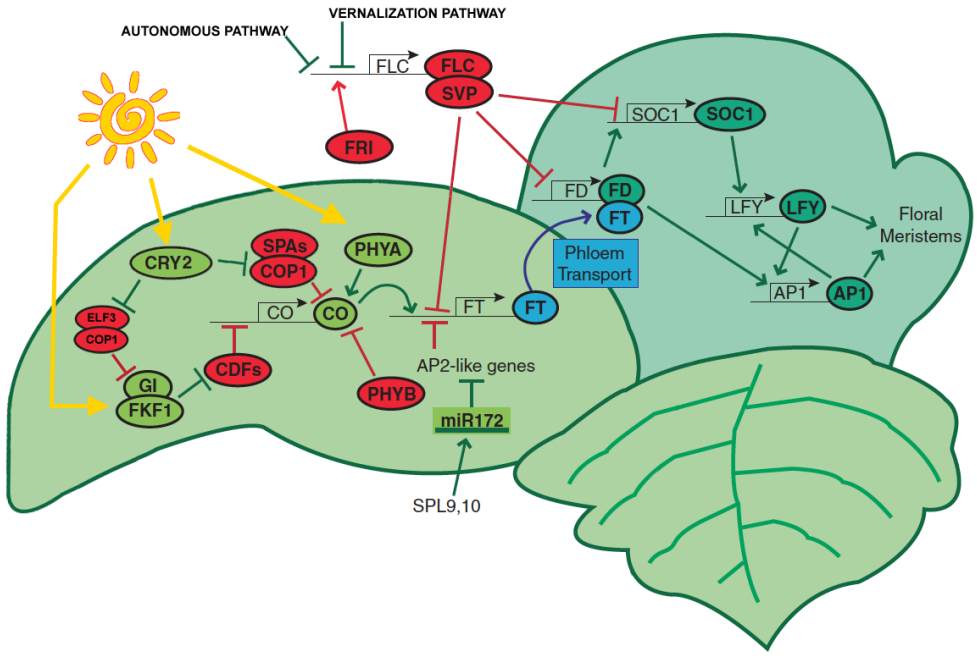

Figure 2. Overview of flowering–time regulation in Arabidopsis.

Genes, proteins (represented as ovals), microRNAs and pathways are described in the text. Solid green or red lines with an arrow represent promotion, and those with a perpendicular bar represent repression. Components that overall promote flowering are shown in green, and those that repress flowering are shown in red (Modified – Amasino 2010).

GI transcript accumulation displays circadian variations (peaking in the middle of the subjective day) (Fowler et al., 1999). GI protein is also controlled at the post-translational levels, being stabilized in the light phase and degraded in the dark (David et al., 2006). Under LDs, at dawn, CRY2 stabilises GI by disrupting the protein complex formed by EARLY

FLOWERING 3 (ELF3, which is a plant-specific nuclear protein with no known functional domains) and the ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Yu et al., 2008). Because COP1 is responsible for GI ubiquitin–dependent degradation, CRY2 action results in the stabilization of the GI protein and the subsequent association of GI with the blue light receptor FKF1 (Sawa et al., 2007). The GI–FKF1 complex promotes the degradation of the CYCLING DOF FACTORs (CDFs, a family of negative regulators of *CO*) thus allowing the upregulation of *CO* mRNA levels at the end of the day (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009). CRY2 also regulates CO protein by preventing its degradation by the SUPPRESSOR OF PHYA-105 (SPA)–COP1 complex (Zuo et al., 2011). Moreover, the phytochromes are involved in post–translations regulation of CO protein levels with PhyA and PhyB playing opposite roles on CO accumulation (Thomas, 2006). CO promotes flowering by upregulating the transcription of the florigens genes *FT* and *TWIN SISTER OF FT* (*TSF*) in the phloem companion cells of the leaves (An et al., 2004; Yamaguchi et al., 2005 and many others).

Photoperiod–dependent, but CO–independent pathways for *FT* upregulation are also present. For instance GI positively regulates the *micro RNA 172* (*miR172*) to trigger post transcriptional gene silencing of a class of APETALA2-like proteins, which are important transcriptional repressors of *FT* in the leaf (Jung et al., 2007). The *miR172* is also under the control of the aging pathway through the action of the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE family (SPLs) (Wu et al., 2009).

From the leaves, where it is induced, the florigen protein moves to the shoot apical meristem (SAM) where floral transition occurs (Corbesier et al., 2007; Jaeger and Wigge, 2007). Upon arrival in the SAM FT interacts with a class of SAM – specific bZIP transcription factors, FD and FDP (Abe et

al., 2005; Wigge et al., 2005). The resulting complex can positive regulate several MADS box type transcription factors, namely *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA1* (*AP1*), and *FRUITFUL* (*FUL*) which initiate the floral transition at the shoot apex (Abe et al., 2005; Wigge et al., 2005).

2.2 The vernalization pathway: the repressive role of FLC

In nature, vernalization defines a process whereby an extended exposure to cold promotes flowering. Vernalization is often associated with the biannual and perennial habit of certain plants, which require an exposure to winter cold to flower in spring (Amasino, 2004). The output of the vernalization pathway is the transcriptional silencing of the floral repressor *FLOWERING LOCUS C* (*FLC*) that in association with SHORT VEGETATIVE PHASE (SVP) prevents flowering by repressing several floral genes including *FT*, *TSF*, *FD* and *SOC1* (Fig. 2) (Hepworth et al., 2002; Helliwell et al., 2006). Before the exposure to cold *FLC* transcription is positively regulated by *FRIGIDA* (*FRI*), which encodes a protein of unknown biochemical function (Johanson et al., 2000) (Fig. 2). Upon exposure to cold temperature *FLC* repression starts with the transcription of its complete antisense mRNA called *COOLAIR* and by the sense transcription of its first intron called *COLDAIR* (Fig. 3) (Swiezewski et al., 2009; Heo and Sung, 2011). After this early response, VERNALIZATION INSENSITIVE 3 (VIN3) methylates the lysine residues of histone H3 of *FLC* chromatin (Fig. 3) (Bond et al., 2009). This marks the epigenetic gene silencing that is mitotically stable and maintained under warm temperature conditions by VERANLIZATION1 (VRN1, a DNA-binding protein) and VRN2 (a

homologue of one of the polycomb group proteins) (Fig. 3) (Gendall et al., 2001; Levy et al., 2002). Thus, FLC epigenetic silencing releases repression at the floral promoting genes and enables the floral transition.

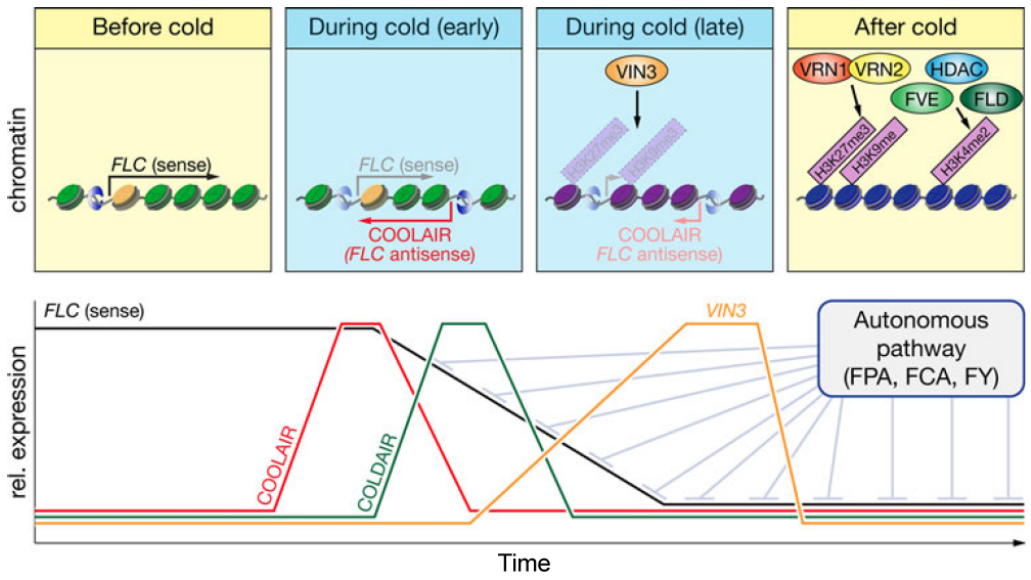

Figure 3. The transcriptional regulation of FLC.

The effect of cold temperature on *FLC* chromatin and transcription is shown. In the early stage of cold repression of *FLC* is achieved through the transcription of *COOLAIR* and *COLD AIR* non coding RNAs. *FLC* repression is stabilized and made permanent by VIN3 VRN1, VRN2. Members of the autonomous pathway, FVE, FLD, FPA, FCA, FY contribute to enhance *FLC* repression (Srikanth and Schmid 2011).

2.3 The autonomous pathway

Typical autonomous pathway genes are *LUMINIDEPENDENS* (*LD*), *FCA*, *FY*, *FPA*, *FLOWERING LOCUS D* (*FLD*), *FVE*, *FLK*, and *REF6* and mutations in any of these genes delay flowering irrespectively of day length (Kim et al., 2009; Srikanth and Schmid, 2011). They encode proteins that

can be divided into two functional categories: general chromatin remodelling factors and proteins that affect RNA processing (Kim et al., 2009; Srikanth and Schmid, 2011). Despite their different functions, they promote flowering by downregulating the floral repressor *FLC* at different levels, epigenetic or post-transcriptional (Fig. 3). In general the late flowering phenotype of autonomous pathway mutants can largely be explained in terms of elevated *FLC* levels and can be reverted by vernalization.

2.4 The Gibberellins pathway

Gibberellins (GAs) are plant–specific hormones required for plant growth by mainly promoting cell elongation and marginally cell division. Moreover, GAs promote different developmental switches, like germination, the juvenile to adult phase change and the transition from vegetative to reproductive development (Mutasa-Göttgens and Hedden, 2009). GAs are essential for floral induction under SDs, whereby under these condition strong GA biosynthetic mutants (such as *GA1*) fail to flower. However, under LDs conditions the effect of GAs on flowering is less pronounced (Wilson et al., 1992). GAs accelerate flowering under SDs by activating the transcription *SOC1* and *SPLs* at the shoot meristem and under LDs upregulating of *FT* in the leaves (Porri et al., 2012).

2.5 Emerging pathways fine-tuning the floral transition

The four main flowering pathways regulate flowering time according to major environmental cues. However, in the past few years, physiological and molecular studies have revealed new and/or partially overlapping signalling mechanisms that participate in the regulation of floral transition upon several environmental conditions. For instance, ambient temperature is a key factor affecting the floral transition whereby plants flower earlier when grown at higher temperatures, such as 28°C, compared to lower temperatures. The bHLH transcriptional regulator PHYTOCHROME INTERACTING FACTOR 4 (PIF4) is responsible for mediating this floral acceleration through the upregulation of *FT (*Kumar et al. 2012). In contrast, at lower temperatures, such as 16°C, flowering is delayed (Balasubramanian et al., 2006). It has been suggested that SVP is involved in the cold–dependent floral delay by the repression of FT (Lee et al., 2007). In addition to ambient temperature, flowering time in plants is strongly affected by other environmental stresses such as salinity in the soil (Achard et al., 2006) and nutrient availability (Kant et al., 2011).

Water availability is necessary for plant growth and yield. Indeed water scarcity is one of the most important abiotic stresses limiting crop production in different parts of the world. Independent evidences, based on flowering time measurements in different plant species, indicate that drought stress causes an acceleration of flowering (known as drought escape). Since the mechanisms underling this phenomenon is currently unknown, the study of the model plant Arabidopsis may inform us on its molecular components, a key goal of my thesis.

3. Water scarcity

Plants can cope with drought stress by altering their physiology and morphology to survive and to sustain growth. Water scarcity responses have been grouped by Ludlow (1989) into three strategies: dehydration tolerance, dehydration avoidance and drought escape. Dehydration tolerance is a typical strategy adopted by plants in extremely arid environments. They accumulate protective metabolites and proteins and undergo a drastic reduction in metabolic activity thus entering into a dormant or semi-dormant state, which allow them to survive severe stress. Dehydration avoidance takes place under a moderate stress condition. This is the most common strategy whereby plants try to maintain the internal water status by stomatal closure (to minimize water loss), increased root–to–shoot ratio (to maximize water uptake) and solute accumulation. Drought escape refers to as the ability of plants to adjust and complete their life cycle before severe stress sets in (Meyre et al., 2001; Verslues and Juenger, 2011). These strategies are carried out by involving complex physiological mechanisms and gene networks alterations and are often used in concert to survive water deprivation.

The plant hormone Abscisic Acid (ABA), acts as a key endogenous messenger in response to different environmental stress such as drought. It has been shown that drought triggers ABA accumulation in plants, which in turn causes stomatal closure and induces the expression of stress-related genes (Christmann et al., 2005).

3.1 ABA biosynthesis

ABA derives from carotenoids synthesized in plastids. The first step of ABA biosynthesis is the transformation zeaxanthin into violaxanthin by the *ABA1* gene, encoding a zeaxanthin epoxidase that transforms the zeaxanthin into violaxanthin. After that, through the action of *ABA4* and *NCED* genes, the violaxanthin is converted into xanthoxine. The last steps of the ABA biosynthesis occur in the cytosol via the action of the *ABA2* and *ABA3* genes.

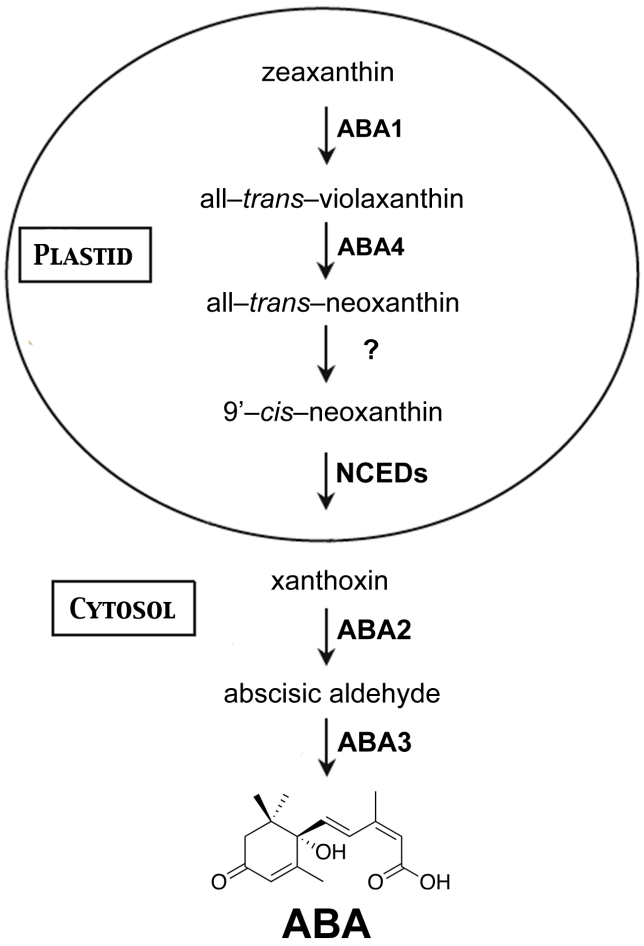

Figure 4. ABA biosynthesis.

The biosynthetic pathway from zeaxanthin is shown, with identified enzymes as indicated and described in the text. Unidentified steps are indicated with a question mark (?).

3.2 Mechanisms of ABA signalling

ABA controls several adaptive responses to environmental stresses as well as growth and development process including seed maturation and seed dormancy. Three proteins represent the key signalling nodes underlying ABA signalling. These are the *PYRABACTIN RESISTANCE* (PYR)/*REGULATORY COMPONENT OF ABA RECEPTOR* (RCAR), the Protein Phosphatase 2Cs (PP2Cs), and SNF1-related protein kinase 2s (SnRK2s). Park et al. (2009) and Ma et al. (2009) have shown that the PYR/RCARs act as an ABA receptor, the PP2Cs act as negative regulators of the pathway, and SnRK2s act as positive regulators of downstream signalling. Upon perception of a stress signal, ABA is induced primarily in the vascular tissue in Arabidopsis. ABA binds to the ABA receptor (RCARs/ PYR1 /PYLs) and this complex inhibits the action of group A PP2C proteins (such as ABI1 and ABI2) which act as negative regulators of the pathway. In the absence of ABA, PP2Cs dephosphorylate SnRK2s thus preventing their activity. In agreement with this model, suppression of PP2Cs activities allows the activation of SnRK2s and constitutive ABA signalling (Park et al., 2009; Umezawa et al., 2009).

As described above SnRK2s act as positive regulators of downstream ABA signalling. Several SnRK2s targets have been identified both at the plasma membrane and in the nucleus. SnRK2s are involved in the direct phosphorylation of several bZIP transcription factors (ABFs) in the nucleus to promote ABA–dependent gene expression (Johnson et al., 2002; Furihata et al., 2006; Fujii et al., 2007). SnRK2s control the phosphorylation state of a two classes of anion and cation channels at the plasma membrane to regulate stomata movements (Mustilli et al., 2002; Geiger et al., 2009; Sato et al., 2009).

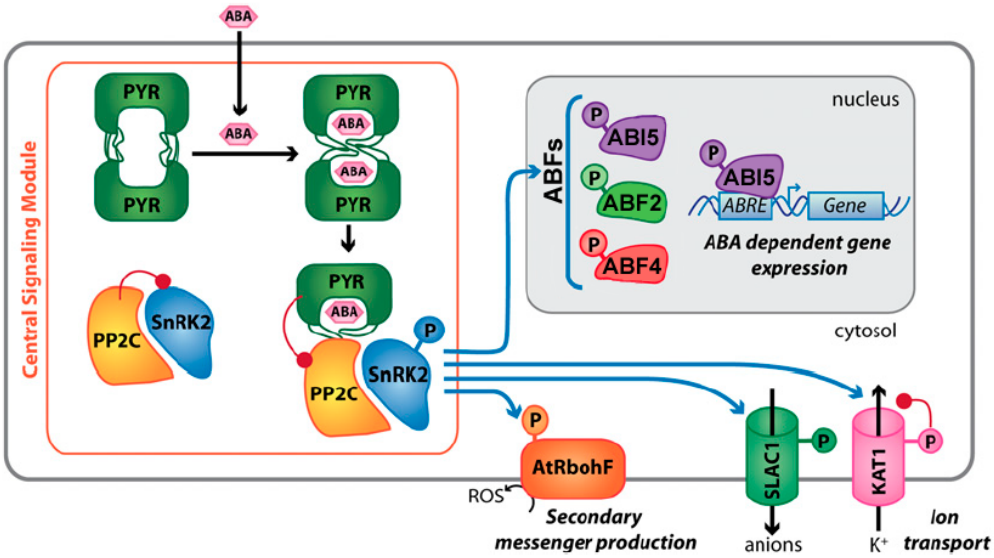

Figure 5. The core ABA signalling pathway.

Recent progresses in our understanding of the early ABA signalling events have led to the construction of a PYR/RCAR–PP2C–SnRK2 signal transduction model. In the absence of ABA, PP2Cs inhibit SnRK2s–type protein kinases. ABA is bound by intracellular PYR/PYL dimers, which dissociate to form an ABA receptor–PP2C complexes. Complex formation inhibits the activity of the PP2Cs in an ABA-dependent manner, allowing activation of the SnRK2s. Several SnRK2s targets have been identified both at the plasma membrane and in the nucleus, resulting in control of ion channels, secondary messenger production, and gene expression. Red connections on left indicate an inhibitory interaction (Modified – Hubbard et al., 2010).

Aim of the Project

Plants can cope with drought stress using different strategies, some of them are well studied other, like drought escape (DE), are poorly understood. The DE response is an adaptive strategy by which plants modify their life cycle, accelerating their flowering time to avoid stress and to produce a progeny before death.

The aims of this thesis work are to define the presence of the drought escape (DE) response in *A. thaliana,* to understand its genetic basis, and describe a possible mechanism to explain how drought signals are integrated into the floral transition.

To achieve these goals three related lines of research were undertaken. First, I aimed at developing the experimental conditions to robustly evoke a DE response in Arabidopsis. Subsequently, to understand the genetic basis of the DE response I screened flowering time mutants for their ability to produce a DE response. Third, mutants with altered DE response behaviour (likely components of the mechanism allowing DE response) were studied in more detail to understand their mode of action and drought dependent regulation.

Main Results – Discussion

4. The role of photoperiod in mediating DE in Arabidopsis thaliana

In this work we used a genetic approach to dissect the drought escape (DE) response in the widely used laboratory strains of *A. thaliana,* Landsberg *erecta* (L*er*) and Columbia (Col-0). We demonstrate that DE can only occur under long days (LDs) photoperiods, whilst under short days (SDs) drought stress causes a delay in flowering.

Several components of the photoperiodic pathway are necessary to trigger a DE response, namely *GIGANTEA* (*GI*), *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). GI, in association with three photoreceptors (FKF1, ZTL and LKP2), allows plants to measure day length (Kim et al., 2007; Sawa et al., 2007; Baudry et al., 2010). However, GI affects different biological processes, perhaps independently of its well-established role in day length. For instance GI is involved in the regulation of starch production, oxidative and salinity stress responses, as well as cold tolerance (Eimert et al., 1995; Kurepa et al., 1998; Cao et al., 2005; Kim et al., 2013). *gi* mutants show a general increase in the accumulation of a wide range of sugars (Messerli et al., 2007). Higher level of osmo–active solutes could alter the perception of the stress. Thus, establishing the nature of GI–signalling in the context of the DE response remains an open question.

Our transcript analysis on well-established ABA– dependent and independent markers suggests an altered perception of drought stress by *gi* mutants (Fig. 7 – Part III). In particular we found that the transcription of

ABI2, was completely abolished in the *gi* background while *RD29a*, *CBF3* and *KIN1* transcript levels were similar to wild type under normal watering conditions but were not upregulated upon drought stress (Fig. 7A and C – Part III). In contrast *COR15a* and partially *KIN1* were constitutively upregulated compared to wild type (Fig. 7A and C – Part III). We interpret such transcriptional alterations as an additional clue for GI having a role in stress signalling. Despite these observations, it is worth noticing that the drought dependent upregulation of these markers did not evidently appear to be under a photoperiodic control (with the possible exception of *ABI2*) (Fig. 7A – Part III). Therefore the role of GI in the transcription of these genes may be largely separate from photoperiod. We thus favour a model where photoperiod–activated GI protein triggers a DE response. This hypothesis is mainly based on the observation that the DE response of *gi* mutants can be effectively phenocopied in wild type plants grown under SDs (Fig. 1A, B and 2A, B – Part II). Thus, lack of DE in *gi* mutants may be more likely attributed to a lack in day length information, rather than a difference in starch accumulation or perturbation the stress signalling. Ultimately, the targets of GI are the florigen genes *FT and TFS*, which are strongly upregulated under drought conditions (Fig. 4A, B – Part II). However, because no change in *GI* transcript occur upon drought stress (Fig. S4C – Part II), we hypothesize that drought may affect *GI* signalling downstream of its transcription.

Our data suggest a novel mode of GI action for florigen upregulation under drought conditions. According to the current understanding of photoperiodic signalling, GI forms a complex with a class of photoreceptors – F–box proteins (namely FKF1, ZTL and LKP2) in a light–dependent manner and this interaction triggers downstream signalling. FKF1 in association with GI regulates the transcription of *CO* through the degradation of CDFs

transcriptional repressors (Imaizumi et al., 2005; Sawa et al., 2007). Although the main GI–dependent *FT* regulation relies on the FKF1–CO module or the mir172 pathway (Jung et al., 2007), recently Sawa et al. (2011) have shown that the GI–FKF1 complex can directly bind the *FT* promoter. However *fkf1* and *ztl* single and *fkf1 ztl lkp2* triple mutants are not defective in DE response (Fig 1A, B – Part III; 8A, B – Part III). Since DE is a photoperiodic–dependent process it must rely on a photoreceptor, although the identity of this presumed photoreceptor protein as well as its mechanism of action in concert with GI remains so far elusive. The only photoreceptor mutant showing altered (reduced) DE response is *cry2* (Fig 1A, B – Part II). It has been shown that *CRY2* is co–expressed with the florigens in the vascular bundles where it mediates their transcription via association with CIB1 (CRY-interacting basic-helix-loop-helix) in a blue light dependent manner (Endo et al., 2007; Liu et al., 2008). CRY2 also indirectly affects the stability of GI by repressing the COP1–ELF3 complex (which degrades GI) and in association with SPA proteins it contributes to stabilise CO protein (Yu et al., 2008; Zuo et al., 2011). Moreover CRY2 regulates the stability of HY5, a bZIP protein that participates in both light and ABA signalling during germination through the ABA – dependent transcriptional activation of *ABI5* (Osterlund et al., 2000; Chen et al., 2008). All these evidences coupled with its defective DE response suggests that CRY2 could be a key component in connecting and integrating light and ABA signalling, although whether it can also mediate photoperiodic cues in association with GI is currently unknown.

A delay in flowering upon drought was observed in wild–type plants under SDs or in *gi* and *tsf ft* plants under LDs (Fig. 1A, B and 2A, B – Part II). Thus in the absence of florigen activity drought stress produces a repression of flowering. Since a drought–mediated floral delay occurred

also in ABA deficient mutants suggests that drought delays flowering (also) in an ABA–independent manner (Fig. 3A, B – Part II). We do not know where such negative regulation of flowering by drought takes place. One possible scenario is that drought exerts a negative role directly in the SAM. Such negative role can be largely overcome under LDs, upon migration of the florigen protein in the SAM. Likely candidate genes negatively regulating flowering in a drought dependent manner include the floral repressors *FLC*. Indeed *FLC* is strongly upregulated in the *hab1–1 abi1–2 pp2ca–1* mutants, reflecting their late flowering phenotype under SDs (Fig. 3B – Part III; 3E – Part II). More work is required to clarify the spatial regulation of ABA signalling in flowering, including mis-regulation experiments (e.g. by altering ABA signalling in different districts of the plant including the leaf, or the SAM itself). Our working hypothesis is that LDs might promote the interaction between GI and some repressors complex at the florigen promoter, thus enabling its drought dependent induction.

5. The role of ABA in the floral transition

The phytohormone ABA mediates different developmental and stress responses, principally those connected to the drought stress (Fujita et al., 2011; Huang et al., 2012; Sreenivasulu et al., 2012). During the last twenty years ABA as ben proposed as a general inhibitor of the floral transition, although in some cases it appears to be a promoter. One of the first report suggesting a repressive role for ABA in flowering came form a work of Martinez–Zapater that in 1994 showed that the *aba1–1* and *abi1–1* mutants are somewhat early flowering under SDs. Later evidence for an inhibitory role of ABA came from data collected from *in vitro*–grown plants and

exogenous ABA applications. Barrero et al. (2005) showed that *aba1* mutants are early flowering *in vitro* culture under constant light and that exogenous ABA applications inhibit flowering in wild-type plants under long days (Blazquez et al., 1998; Razem et al., 2006; Peters et al., 2010). Domagalska et al. (2010) reported that *aba2* mutants (Wassilewskija background) exhibit a modest early flowering under LDs. Other data from independent mutants impaired in ABA signalling supported an inhibitory role for ABA in flowering. For instance mutants for the β subunit of farnesyltransferase *era1* (*enhanced response to ABA 1*) are late flowering both in LDs and SDs (Yalovsky et al., 2000) despite the exact role of *ERA1* in the ABA signalling cascade is not clear. CBF4 (part of the CBF/DREB1 protein) is a transcription factor involved in ABA signalling and the *35S:CBF4* show a delayed flowering time (Haake et al., 2002). Also *abi3* mutants, a key ABA signalling component, are early flowering in both SDs and LDs and this is accompanied with high levels of *TSF*, suggesting a repressive role for ABI3 on *TSF* expression (Rohde et al., 2000; Kurup et al., 2001).

The role of ABA in flowering is however controversial as different reports suggest also a positive role for ABA during the floral transition. In the paper of Domagalska et al. the author also suggested that transgenic lines that overexpress *NCED3* (a key enzyme in the ABA biosynthesis) are slightly earlier flowering than wild type. Mutations in the gene encoding the large subunit of the nuclear mRNA cap-binding protein, *ABA hypersensitive 1* (ABH1), cause early flowering irrespective of the day length. ABH1 is involved in the regulation of FLC transcription (Bezerra et al., 2004). Moreover, ABA can positively regulate the transcription of *SOC1* through the action of the *OXIDATIVE STRESS 2* (*OX2*) zinc–finger transcription factor family. These proteins are able bind in an ABA–dependent manner

the promoter of *SOC1* and multiple mutants in the *OX2* family are late flowering (Blanvillain et al., 2011). Evidence for the positive role of ABA in flowering also derives from different species. In *Pharbitis nil* grown under 12-h-long subinductive night, applications of ABA on cotyledons results in a floral induction while application of NDGA, an inhibitor of ABA biosynthesis, clearly inhibited flowering when applied during a 16-h-long inductive night (Wilmowicz et al., 2008).

Our data are in accord with a positive role for ABA in flowering. One possible explanation is that previous reports were heavily influenced by data collected from *in vitro*–grown plants and exogenous ABA applications. For instance we also detected an early flowering time phenotype in *aba1* mutants under *in vitro* conditions, which is not observed on soil (Fig. 1A, B – Part III). We similarly find that exogenous ABA application delay flowering but this is likely the result of non–canonical effects derived from the exogenous ABA applications (Fig. 1A, C, D, E and F – Part III). Recent advancements on hormone signalling reveal how their site of production, mode of transport and action is strictly controlled; ABA may thus be no exception.

We find that under LDs ABA accelerates flowering by activating the florigen genes (Fig. 4C – Part II). A positive role for endogenous ABA in controlling the floral transition under LDs derive from three main pieces of evidences; first, the late flowering phenotype of different *aba* mutants (Fig. 3A, C – Part II; 2A, B – Part III). Second their reduced DE response (Fig. 3B – Part II). Third, the reduced transcript levels of the florigens and *SOC1* in *aba* mutants (Fig. 4C – Part II). Also the ABA hypersensitive triple mutant *hab1–1 abi1–2 pp2ca–*1 is early flowering under LDs and transcriptional analysis suggests that *FT* is the principal florigen target of the ABA (Fig. 3A – Part II; 3B – Part III).

Different components of the ABA signalling pathway may be involved in the positive regulation of *FT*. Among them are the ABFs, a bZIP transcription factor family regulated by ABA and mediating downstream ABA responses (Choi et al., 2000; Yoshida et al., 2010). *NF–Y*–type transcription factors interact with ABFs as well as the *FT* primary regulator CO (Kumimoto et al., 2010). Moreover *ABI3* could participate to the repressive role of the ABA since the *abi3* mutants are early flowering irrespectively of the photoperiod qualifying it as a floral repressor(Kurup et al., 2001).

Under SDs drought and ABA are negative regulators of the floral transition. WT plants under drought stress are late flowering and the same occurs in *aba1–6* mutants (Fig. 3E – Part II). This could be explained in terms of residual ABA production in the *aba1–6* mutant or the action of ABA–independent pathways. Under SDs the *hab1–1 abi1–2 pp2ca–1* was extremely late flowering (Fig. 3E – Part II) and had enhanced *FLC* levels compared to WT also in normal watering condition (Fig. 3B – Part III). Supporting a role of ABA in FLC upregulation it has been recently shown by Wang et al. (2013) that *ABI5* (a bZIP transcription factor related to the *ABFs* family) can delay the floral transition through the upregulation of *FLC* in an ABA dependent manner.

Future analysis will clarify how these transcription factors interact, how they are regulated by ABA and their binding mechanism (if any) to the *FT* promoter.

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PART II

Title: *GIGANTEA* enables drought escape response via ABA–dependent activation of the Florigens and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*

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One Sentence Summary:

GIGANTEA is required to accelerate the floral transition under drought conditions by enabling the ABA–dependent upregulation of the Florigen genes.

ABSTRACT

Modulation of the transition to flowering plays an important role in the adaptation to drought. The drought escape (DE) response allows plants to adaptively shorten their life cycle to make seeds before severe stress leads to death. However, the molecular basis of DE response is unknown. The screen of different *Arabidopsis thaliana* flowering time mutants under DE– triggering conditions revealed the central role of the flower–promoting gene *GIGANTEA* (*GI*) and the florigen genes *FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*) in the DE response. Further screens showed that the phytohormone abscisic acid is required for DE response, positively regulating flowering under long day conditions (LDs). Drought stress promotes the transcriptional upregulation of the florigens in an ABA– and photoperiod– dependent manner, so that early flowering only occurs under LDs. Along with the florigens, the floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) is also up-regulated in a similar fashion and contributes to the activation of *TSF*. The DE response was recovered under short days in the absence of the floral repressor *SHORT VEGETATIVE PHASE* (*SVP*) or in *GI* overexpressing plants. Our data reveal a key role for GI in connecting photoperiodic cues and environmental stress independently from the central *FT*/*TSF* activator *CONSTANS*. This mechanism explains how environmental cues may act upon the florigen genes in a photoperiodically–controlled manner, thus enabling plastic flowering responses.

INTRODUCTION

The timing of the floral transition has significant consequences for the reproductive success of plants and consequently their adaptability to various environmental conditions. Plasticity in flowering time in response to changes in water availability has been documented in several plant species (Xu et al., 2005; Lafitte et al., 2006; Sherrard and Maherali, 2006; Franks et al., 2007; Franks, 2011; Ivey and Carr, 2012). As water scarcity results in a reduction of growing seasons, the drought escape (DE) response defines the ability of plants to complete their life cycle before stress conditions deteriorate to lethality (McKay et al., 2003; Verslues and Juenger, 2011). Thus, in natural environments the onset of the DE response represents a key adaptive trait in triggering an acceleration of the floral transition and reproductive success (Franks, 2011). Despite its ecological significance, a DE response has not yet been ascribed to a mechanism of flowering gene regulation. Therefore a key question is what mechanism transduces a drought–derived signal into affecting the floral transition?

The floral transition is controlled by internal and external factors and occurs when the shoot apical meristem (SAM) receives appropriate signals and switches from producing vegetative leaves to producing flowers, fruits and seeds (Bernier et al., 1993). The study of the model plant *Arabidopsis thaliana* resulted in the definition of four major pathways involved in flowering time control: the photoperiodic, the vernalization, the autonomous and the gibberellins (GAs) pathways (Amasino, 2010; Andrés and Coupland, 2012).

Flowering in annual Arabidopsis ecotypes is strongly promoted by long day (LD) photoperiod conditions, typical of spring/early summertime. The

photoperiodic pathway is characterized by three key components, whose regulation and activity is required for correct day length measurement: *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) (Putterill et al., 1995; Fowler et al., 1999; Kardailsky et al., 1999; Kobayashi et al., 1999; Park et al., 1999). Mutations in any of these genes delay flowering under LDs, with little effect under short day (SD) conditions. Day length duration is perceived in the leaves where a systemic signal (known as florigen) originates (Evans, 1971). During LDs, light promotes the interaction between GI and a family of light sensing F–box ubiquitin ligases which results in the degradation of a set of transcriptional repressors at the *CO* promoter (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009). LDs also promote the stabilization of CO protein and the consequent activation of the florigen genes *FT* and *TWIN SISTER OF FT* (*TSF*) in the phloem companion cells (An et al., 2004; Valverde et al., 2004; Yamaguchi et al., 2005; Jang et al., 2009). However, the FT protein moves to the SAM where it interacts with the bZIP transcription factor FLOWERING LOCUS D (FD) to orchestrate the floral transition (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) encodes a MADS box transcription factor and represents an early target of the FT/FD complex in the SAM (Lee et al., 2000; Lee and Lee, 2010).

Mutations in the autonomous pathway cause a delay in flowering irrespective of the photoperiod. The autonomous pathway promotes flowering by downregulating the floral repressor *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999; Michaels and Amasino, 2001). The late–flowering phenotype of autonomous pathway mutants can be reverted by vernalization, which targets *FLC* chromatin by imposing a silenced epigenetic state (Kim et al., 2009). GAs play a key role in flowering,

particularly under SDs since GAs–deficient mutants do not flower under those conditions (Wilson et al., 1992).

In nature, plants are exposed to a variety of external cues with remarkable, yet contrasting effects on flowering. For instance, warm temperatures (28°C) substantially accelerate flowering compared to cool temperatures (16°C) in *Arabidopsis* (Blazquez et al., 2003; Balasubramanian et al., 2006). Abiotic stresses such as UV–C exposure accelerate flowering (Martínez et al., 2004). Conversely, intermittent cold treatment and salt stress inhibit flowering (Achard et al., 2006; Seo et al., 2009). Recent data show the importance of nutrient availability and the opposing role of nitrate and phosphate on flowering (Kant et al., 2011). Thus, plants are able to discriminate the type of external "stress" and to integrate this information into the flowering network. A key goal in flowering studies is therefore to define the mechanistic basis underlying such integration and its physiological significance.

FT is a central node of floral integration since its expression depends on multiple inputs (Pin and Nilsson, 2012). *FT* is mainly controlled in a photoperiodic–manner. However, other external stimuli have been shown to directly converge at the *FT* promoter including blue light and warm temperature (Liu et al., 2008b; Kumar et al., 2012). Besides being positively controlled, *FT* expression is further fine–tuned via modulation of the activity of several repressor complexes including FLC / SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO1 (TEM1) and SCHLAFMÜTZE /APETALA 2–like (Castillejo and Pelaz, 2008; Li et al., 2008; Mathieu et al., 2009).

Warm temperature is arguably the best–characterized paradigm for stress–dependent *FT* upregulation. However, warm ambient temperature triggers *FT* upregulation both under SDs and LDs conditions (Kumar et al., 2012). Here we propose a model for interaction between photoperiod and drought

stress, whereby photoperiod–activated GI enables the ABA and drought – mediated activation of *FT*/*TSF* and *SOC1*. Consequently plants can maximize their fitness by coordinating stress responses according to seasonal cues.

RESULTS

Early drought Stress Triggers DE in Arabidopsis

To assess the presence of a DE response strategy in Arabidopsis and to define the genetic basis underlying this adaptive trait, we set up conditions to impose a persistent drought stress starting from early stages of development. Three day–old seedlings were either watered daily to maintain a relative water soil content (RWSC) at 80–90% or not watered to allow soil moisture to decrease to 30% (Figure S1A). A *bona fide* water stress condition was reached within six days after sowing, as confirmed by the increase in the ABA–dependent markers *ABSCISIC ACID INSENSITIVE 2* (*ABI2*) and *RESPONSIVE TO ABA 18* (*RAB18*) (Lång and Palva, 1992; Nemhauser et al., 2006) (Figure S1B and S1C). These water deficiency conditions, maintained throughout the duration of the experiment, were nevertheless compatible with plant growth and survival and resulted in a robust early flowering response. Compared with normal watering, drought treated Col–0 and L*er* wild–type plants produced fewer vegetative leaves as well as an early bolting time, indicative of DE response (Figure 1A to 1E). The early flowering phenotype was reflected in the early upregulation of the floral markers *LEAFY* and *APETALA1* (Blázquez et al., 1997; Hempel et al., 1997) in plants undergoing drought stress compared with normal watering controls (Figure S1D and S1E).

DE Response Requires GI, FT/TSF and SOC1

To determine whether the DE response observed in wild–type accessions was mediated by any of the known flowering–time genes we imposed DE–triggering conditions under LDs upon different late flowering time mutants that are representatives of all known floral pathways (Figure 1A to 1D).

Mutants in the autonomous pathway (*lumnidependens* – *ld*, *fve*, *fy* and *fca*, which flower late irrespective of the photoperiod) and the gibberellin pathway (*ga1*, impaired in GA production) produced a DE response relatively similar to wild type, as they were consistently early flowering under DE–triggering conditions (Figure 1A to 1D). A complete absence of DE response was observed in *gi* mutants both in the Col–0 (*gi–100*) and L*er* background (*gi–4*) (Figure 1A to 1D and 1F). We confirmed the requirement for *GI* in triggering DE response by analyzing independent alleles of *gi* (*gi–1, gi–2, gi–5* and *gi–6*), ruling out an allele– or ecotype–specific effect (Figure S2A and S2B). Furthermore, *gi* plants also displayed a significant delay in flowering time under a restricted watering regime, but this was more pronounced in the Col–0 background compared to L*er* (Figure 1A to 1D, 1F and Figure S2A and S2B).

Despite the known functional dependence of GI on light–sensing protein interactors such as FLAVIN–BINDING, KELCH REPEAT, F–BOX 1 (FKF1) and ZEITLUPE (ZTL) – responsible for GI– mediated *CO* activation and clock function, respectively (Imaizumi et al., 2005; Kim et al., 2007)– no evident defects in the DE response were found in single *fkf1* and *ztl* mutants (Figure 1A and 1B).

Interestingly, we found that mutants in the blue–light photoreceptor *CRYPTOCHROME2* (*cry2–1,* Col–0 and *fha–3*, L*er*) were significantly impaired in their DE response (Figure 1A to 1D). As CRY2 affects the photoperiodic pathway at different levels, including the promotion of GI

protein stability (Yu et al., 2008; Zuo et al., 2011), this finding may support the central role of GI in mediating DE response.

In accordance with GI being ultimately responsible for the photoperiodic activation of the florigen genes *FT* and *TSF*, *ft tsf* double mutants (but not their respective single mutants) lacked the DE response, largely mimicking the *gi* mutants (Figure 1A to 1D and 1G). Although these data point to a florigen–dependent mechanism for DE activation, this response does not appear to require the activity of *CO*, a transcriptional regulator of *FT* and *TFS* that acts downstream of *GI* in mediating the photoperiodic response. Also, no DE response defects were observed in *phytochrome A* (*phyA*) mutants, which affect CO protein levels (Valverde et al., 2004) and are thus largely downstream of *GI* (Figure 1C and 1D).

GI–dependent but *CO*–independent pathways of *FT* activation have been described (Jung et al., 2007; Sawa and Kay, 2011). One such pathway involves the GI–dependent activation of the *microRNA172* (*miR172*) resulting in the post–transcriptional gene silencing of the *AP2like* genes (a class of *FT* transcriptional repressors) (Yant et al., 2010). If this was the case, we would expect a reduction in the DE response in plants carrying an activation tagged allele of the *AP2–like* gene *SCHLAFMÜTZE* (*smz–D*) (Mathieu et al., 2009). However, *smz–D* plants exhibited an unaltered DE response, suggesting another mode of GI action (Figure 1A and 1B).

Despite the central role of the florigen proteins in mediating the DE response, no defects were found in *fd*, whose wild–type gene product represents a key FT interactor in the SAM (Figure 1A and 1B) (Abe et al., 2005; Wigge et al., 2005). This could be due to *FD PARALOG* (*FDP*), mediating florigen signaling in the SAM redundantly with FD (Jaeger et al., 2013).

A strongly reduced DE response was present in *soc1* plants (*soc1–1*, L*er* and *soc1–2*, Col–0), but not in *fruitfull* (*ful*), both related MADS box type transcription factors and downstream targets of FT in the SAM (Figure 1A to 1D and 1H) (Gu et al., 1998; Samach et al., 2000). Previously it was shown that mutations in *AGAMOUS–LIKE 24* (*AGL24*), a SOC1 interactor and regulator, aggravated the *soc1* mutant flowering phenotype suggesting partial redundancy between these two genes (Lee et al., 2008; Liu et al., 2008a). However, no DE response defects were apparent in *agl24* single mutants and *soc1 agl24* were indistinguishable from *soc1* mutants with respect to their DE response (Figure 1A and 1B). Also, while *gi soc1* double mutants were later flowering than *gi*, they were similar in their lack of DE, suggesting that *GI* and *SOC1* were largely operating in the same pathway in the context of DE response (Figure 1A and 1B). Taken together, our data reveal a co–option of *GI*, but not *CO*, to activate DE response in a florigen – and *SOC1* – dependent manner.

The Onset of the DE Response is Photoperiod–Dependent

We analyzed the DE phenotype of plants grown under SDs to test its photoperiod–dependency. In contrast to LDs, wild–type plants (L*er* or Col–0) did not generate DE response under SDs (Figure 2A, 2B, 2E and 2G). Interestingly, SDs–grown Col–0 wild–type plants (but not L*er*) produced a significant delay in the floral transition under drought conditions compared with normal watering, reminiscent of that previously observed in *gi* or *ft tsf* mutants under LDs. Thus, the DE response appears to be dependent upon GI mediating LD photoperiodic cues, a finding that prompted us to test whether artificial ectopic expression of *GI* would be sufficient in restoring the DE response under SDs. *35S:GI*, (L*er*), or *35S:HA–GI*

(*HEMAGGLUTININ–GI*, Col–0) recovered the DE response, supporting the photoperiod–dependency model for DE activation (Figure 2A, 2B and 2H).
Under LDs *35S:GI* and *35S:SOC1* plants did not display a DE response. This could be due to their early floral transition, occurring before the perception of any significant drought stress stimulus (Figure 2D). *35S:SOC1* plants did not recover the DE response under SDs, exhibiting early flowering irrespective of the irrigation conditions (Figure 2A, 2B and 2C). Double hemizygous *35S:GI / - 35S:SOC1* / - plants under SDs were earlier than their respective parental lines (Figure 2A and 2B) but did not produce the DE response, further indicating that *SOC1* action is downstream of *GI* in the context of DE response activation. High levels of *SOC1* may thus saturate the floral induction process independently of LDs, thus resulting in a lack of DE response. On the other hand the partial reactivation of the photoperiodic response resulting from *GI* over–expression is sufficient to reinstall the DE response, even in the absence of favourable photoperiodic cues.

DE Response Recovery Under SDs in *svp* Mutants

Drought stress can only promote flowering under LDs via a florigen–dependent mechanism. We therefore hypothesized that by relieving the repressive state at the promoter of the florigen genes we could restore the DE response under SDs.
Several *FT* repressors have been characterized, namely the gene products *FLC*, *FLOWERING LOCUS M* (*FLM*), *AP2like* (e.g. *SMZ*, *SCHNARCHZAPFEN – SNZ*, *TARGET OF EAT 1* and *2 – TOE1* and *2*) and *SVP* (Yant et al., 2009). Under LDs the effect of *flc* and *flm* mutations did not appear to alter the DE response (Figure 2C and 2D). In contrast, no significant DE response occurred in *svp* mutants, which exhibited an

extremely early flowering phenotype, independent of the irrigation regime (Figure 2C and 2D).

Under SDs no DE was observed in *flm* or *smz snz toe1 toe2* mutants (Figure 2A and 2B and Figure S3A and S3B). As *SMZ* requires *FLM* to exert its repressive function on *FT* (Mathieu et al., 2009) these data indicate that the *SMZ*/*FLM* transcriptional repressor complex is not responsible for the lack of DE response under SDs. Rather, our results indicate an important role for the FLC/SVP complex in preventing the DE response under SDs. As expected, *flc* mutants were slightly earlier flowering under SDs compared with wild type (Figure 2A, 2B and Figure S3A and S3B). However, unlike wild–type, *flc* plants did not exhibit a floral delay when grown under drought conditions. Interestingly, *svp* plants were able to recover a strong DE response under SDs (Figure 2A, 2B and 2F).

Although lacking the DE response, L*er* wild–type plants did not exhibit a flowering delay under drought conditions when grown under SDs (Figure 2A, 2B and 2G). The fact that the L*er* ecotype carries a weaker allele of *FLC* compared with Col–0 (Lee et al., 1994), coupled with the lack of a floral delay in *flc* mutants (Col–0 background) under SDs could account for this observation. In support for this hypothesis, *fca* and *fve* mutants –L*er*, characterized by increased levels of *FLC* (Sheldon et al., 2000)– produced a significant floral delay under drought conditions compared with normal watering (Figure 2A and 2B). Noticeably, compared to *fca*, *fve* plants exhibited a more pronounced floral delay, which correlates with the high levels of *SVP* being present in this particular genotype (Li et al., 2008).

Drought–induced changes in *FLC*/*SVP* transcript levels could account for such floral delay. *FLC* transcript levels (but not *SVP*) were slightly, but reproducibly increased under drought conditions in both LDs and SDs (Figure S4A and S4B). However, such increment in *FLC* transcript levels is

unlikely to play a significant role under LDs as *fve*, *fy, ld* and *fca* plants did not exhibit obvious DE defects (Figure 1A to 1D). Also, plants ectopically expressing *SVP* (*35S:SVP*) under LDs did not exhibit DE defects (Figure 2C and 2D).

Taken together these data indicate that SVP, likely in association with its interactor FLC, contributes to prevent the DE response upon drought conditions under SDs. Conversely, LD conditions overcome the FLC/SVP repression largely post–transcriptionally to enable the DE response.

The Phytohormone ABA Promotes the DE Response under LDs and Affects Flowering in a Photoperiod–Dependent Manner

The phytohormone ABA plays a pivotal role in orchestrating several drought responses but its role in flowering time is poorly understood (Fujita et al., 2011). Mutants impaired in ABA biosynthesis *aba deficient 1* and *2* (*aba1–6* and *aba2–4*) flowered later than wild type even under normal watering conditions, indicating a positive role for ABA in controlling the floral transition (Figure 3A and 3C). Despite being significantly later flowering than wild–type, *aba2–4* plants were consistently earlier than *aba1–6* (Student's t test, P = 0.02), which could reflect the relative severity of this particular allele.

Under drought stress conditions *aba1* mutant plants exhibited reduced DE response compared with wild type (Figure 3A and 3B). However, because of the residual DE response in *aba1* mutants, other non–ABA dependent pathways are likely to contribute to the early flowering phenotype caused by drought. Alternatively, residual ABA production in these mutants (Ethyl methanesulfonate–generated, nucleotide substitution alleles and unlikely to be null) was sufficient to generate the DE response. To distinguish between these possibilities we analyzed an *ABA1* T–DNA insertion line (Morris et al.,

2006), which could represent a more severe allele. These *aba1* mutants showed a late flowering phenotype, similar to the *aba1–6* allele under normal watering conditions (Figure S5). However, unlike *aba1–6* plants they could not survive under drought stress conditions, thus precluding an evaluation of their DE response.

To further confirm such positive role of ABA in flowering we analyzed the phenotype of higher order mutants in the ABA negative regulator PP2C phosphatase gene family, known to result in hyper–sensitized ABA signalling (Rubio et al., 2009). Compared with wild type, *hypersensitive to aba1* (*hab1–1*) *aba insensitive 1* (*abi1–2*) *aba insensitive 2* (*abi2–2*) or *hab1–1 abi1–2 protein phosphatase 2ca* (*pp2ca–1*) mutants were significantly earlier flowering, even under normal watering conditions (Figure 3A). Under drought stress conditions their DE response was relatively similar to wild type, likely as a result of the combined contribution of increased ABA accumulation and increased sensitivity (Figure 3B). In agreement with the floral promotive role of ABA under LDs, the early flowering of *hab1–1 abi1–2 pp2ca–1* plants was accompanied by strongly increased *FT* (but not *TSF*) transcript accumulation (Figure 3D).

We hypothesised that the constitutive activation of ABA signaling might overcome the lack of DE under SDs. However, *hab1–1 abi1–2 pp2ca–1* plants were significantly later flowering compared with wild type (producing more than 20 vegetative leaves) under normal watering regimes (Figure 3E). *FLC* levels (but not *SVP*) were elevated in SDs–grown *hab1–1 abi1–2 pp2ca–1* compared with wild type, which could contribute to the phenotype observed (Figure 3G). In contrast, ABA biosynthesis – defective mutants (*aba1–6*) did not exhibit altered flowering time compared with wild type (Figure 3E). Under drought conditions both ABA constitutive signaling or

biosynthesis mutants generated a flowering delay, which was similar to wild type (Figure 3F).

Our results indicate that ABA acts as a positive regulator of flowering under LD conditions, but suppresses flowering under non–inductive SDs.

ABA Upregulates *FT*/*TSF* and *SOC1* Expression in a Photoperiod–Dependent Manner

We sought to precisely monitor the expression of flowering genes in DE–defective genotypes. Normally irrigated – or drought stressed – plants were grown under SDs and then shifted to LDs to allow DE response. Upon a LD shift, in wild–type plants *FT* and *TSF* transcripts levels strongly increased at dusk coinciding with the first and second photo extension periods (Figure 4A). Under drought conditions *FT* and *TSF* upregulation was dramatically increased compared with normally–watered controls especially during the second LD (Figure 4A). Consistent with DE occurring in coincidence with LDs, no obvious *FT* or *TSF* transcript increases were detectable under SDs, irrespective of watering regime (compare Figure 4A with Figure 4F). This was further confirmed by the lack of *FT*/*TSF* upregulation in *gi* mutants despite the transfer to LDs (Figure 4B). It is unlikely that the higher florigen transcript accumulation under drought stress derived from increased GI levels as little variations in *GI* gene expression were apparent at any time point during the experiment, independent of the irrigation regimes (Figure S4C). Rather, the boost in *FT* and *TSF* expression was strongly ABA–dependent as it was nearly abolished in *aba1–6* plants (Figure 4C). Moreover, we found that *aba1–6* had generally reduced photoperiod–dependent upregulation of *FT* and *TSF* transcript levels compared with wild type under normal watering conditions, especially upon the first photo–extension period. Thus, ABA promotes flowering by contributing to florigens

transcript accumulation and by potentiating florigens levels under drought conditions.

Upon a shift to LDs conditions *SOC1* transcripts were also up–regulated in a drought–dependent manner in wild–type plants (Figure 4A). Such upregulation was abolished in *gi* mutants, suggesting that it was mediated by the photoperiod (Figure 4B). We then established that *SOC1* upregulation under drought conditions required ABA and that ABA was also necessary for maintaining wild–type *SOC1* transcript levels even under normal watering conditions (Figure 4C). Thus, similarly to the florigen genes, *SOC1* is subjected to both ABA and photoperiod transcriptional control.

FT positively regulates *SOC1* expression (Michaels, 2005; Yoo et al., 2005) and is responsible for *SOC1* upregulation in the SAM (Jang et al., 2009). Other floral integrators and *FT* targets are up–regulated in the SAM namely *FUL* and *AGL24*, but these did not display a strong drought–dependency in their expression (Figure S4D and S4E). *SOC1* is also expressed in leaves before the floral transition and could play a role in *FT* activation (Lee et al., 2000; Samach et al., 2000; Searle et al., 2006). The observed drought–dependent *SOC1* upregulation occurred very early after the LD shift and therefore it is unlikely to reflect varying *SOC1* levels in the SAM (Figure 4A). In *soc1* mutants grown under normal watering conditions the expression levels of *TSF* (but not *FT*) were generally lower than in wild type (Figure 4D). Under drought conditions *soc1* mutants exhibited strongly reduced *TSF* upregulation but no obvious change in *FT* expression. Thus, besides acting downstream of the florigen in the SAM *SOC1* also acts upstream of the *TSF* gene, possibly conveying an ABA–dependent signal. As previously observed *FT* activation is independent of *SOC1* (Searle et al., 2006), but still strongly ABA–dependent. In support of this model of ABA

independently acting on *FT* and *SOC1*, *aba1 soc1* plants were later flowering than *soc1* single mutants, indicating that ABA deficiency can delay flowering through pathways other than *SOC1* (i.e. *FT*) (Figure 4G).

SVP has been shown to negatively regulate *FT* and *SOC1* expression (Li et al., 2008; Jang et al., 2009). Because *svp* mutants recovered the DE response under SDs we anticipated a photoperiod independent upregulation of the florigens and/or *SOC1* upon drought conditions in the *svp* mutants. Compared with wild type, the levels of *FT* were higher (up to 5 fold) in normally–watered *svp* plants under the short day part of the experiment (Figure 4E and 4F). However, no strong *FT* upregulation occurred at these time points upon drought conditions. Unlike *FT*, *TSF* levels did not greatly differ in *svp* mutants compared with wild type under normal watering, but they were increased upon drought conditions (Figure 4E and 4F). However, this *TSF* upregulation was relatively small if compared to the changes in *TSF* transcript levels occurring under LDs in wild–type plants (Figure 4A). Under normal irrigation *SOC1* transcript levels were strongly increased in *svp* plants under SDs, resembling those observed in wild type under LDs (Figure 4A and 4E). Strikingly, under drought conditions the levels of *SOC1* were further increased, implying that SVP normally prevents the drought–dependent activation of *SOC1* under SDs (Figure 4E). As expected, upon shift to LDs, *svp* plants exhibited a dramatic *SOC1* and florigen genes upregulation compared with wild type. Moreover such upregulation was further boosted under drought conditions (Figure 4E).

In summary, *svp* mutants recover the DE response under SDs and this is reflected in *SOC1* drought–dependent upregulation, but not *FT* and marginally *TSF*. To substantiate the involvement of ABA in mediating this drought–dependent signal in *svp* plants, we generated *aba svp* double

mutants. Under LDs these plants were slightly, but significantly later flowering than *svp* single mutants (Student's t test, P = 0.02) (Figure 4G). This could suggest that the contribution of ABA to flowering in the *svp* mutant background was additive, and largely masked by the strong photoperiod–mediated activation of *FT*. However, under SDs, *aba svp* plants were much more late flowering than *svp* single mutants. This finding is consistent with the idea that under SDs the ABA promotive role on flowering genes (e.g. *SOC1*) is normally impaired due to SVP repression (Figure 4H).

DISCUSSION

Role of GI in DE Response

In this work we identified GI as a key component mediating DE response in Arabidopsis. However, a key question emerges as to what kind of signal is GI transducing to activate the DE response. In the simplest scenario GI mediates day length, effectively enabling the superimposition of drought/ABA stimuli upon the *FT*/*TSF* promoters when day length is favorable. The fact that DE is absent under SDs (phenocopying *gi* mutants under LDs) is in accord with this model. However, GI mediates different signaling pathways that could directly affect drought stress perception and/or responses perhaps independently of its photoperiodic role. *gi* mutants were shown to be hyper tolerant to oxidative stress, to be insensitive to salt–mediated floral delay and to be primed for cold tolerance (Kurepa et al., 1998; Cao et al., 2005; Seo et al., 2009; Kim et al., 2013). In addition *gi* mutants exhibit an enhanced starch accumulation, a relevant aspect to consider in the light of recent data highlighting the importance of starch metabolism and carbon signaling in flowering (Eimert et al., 1995;

Wahl et al., 2013). However, the contribution of starch accumulation in ameliorating drought stress is currently poorly understood (Harb et al., 2010). Intriguingly, *FT* and EARLY FLOWERING 3 (a target and an interactor of GI, respectively) have been recently involved in the control of guard cells activity (Kinoshita et al., 2011). Taken together these observations may suggest a more complex model whereby GI mediates stress stimuli in concert and/or downstream of its photoperiodic role. Perhaps *gi* plants have a constitutive drought tolerant phenotype (e.g., as a result of reduced *FT* expression in stomata), which alter their perception of drought stress. A future goal will be to investigate these possible mechanisms of GI action and to establish their relationship (if any) with the photoperiod.

Although we could not identify the exact role of GI action within the DE response, our expression data indicate that photoperiod–stimulated GI activity is essential for the upregulation of *FT*/*TSF* genes expression under drought stress (Figure 4A and 4B and 5). We therefore anticipate that the underlying mechanism will be different from other modes of environmental upregulation of *FT/TSF* (e.g. warm ambient temperature), which can occur independently of photoperiodic cues (Balasubramanian et al., 2006; Kumar et al., 2012). The precise biochemical function of GI protein is still largely unknown as it was found in association with different protein complexes, thus arguing against a single mode of action. GI activates flowering mainly through the CO–FT module although it can also promote flowering independently of these genes (Kim et al., 2005; Mizoguchi et al., 2005; Jung et al., 2007; Sawa and Kay, 2011). GI has been shown to physically interact with different floral repressors including SVP, FLC and TEM and to directly bind to the *FT* promoter, providing a CO–independent mode of *FT* activation (Sawa and Kay, 2011). Thus, under LDs GI may promote DE

response by regulating chromatin accessibility and/or interfere with repressors activity at the florigen promoters, so to allow their ABA–dependent upregulation (Figure 5). Whether this model can be also applied to *SOC1* activation is still unclear as *SOC1* upregulation under drought conditions may largely derive from increased florigen levels. The observation that *ft tsf* double mutants are unable to trigger a DE response argues in favor of a florigen–dependent mechanism of *SOC1* activation under drought conditions.

Our results highlight the importance of the SVP/FLC complex in preventing the DE response under SDs, but this was not reflected in the recovery of *FT* and *TSF* drought–dependent upregulation (Figure 2A and 2B). This suggests the involvement of additional transcriptional repressors at the florigens promoter, hindering their ABA responsiveness (Figure 5). Rather, the loss of SVP/FLC activity recovered the ABA–dependent *SOC1* upregulation (Figure 5). Accordingly, the early flowering phenotype of *svp* mutants was strongly attenuated under SDs in the *svp aba1* double mutants suggesting that SVP normally prevents ABA from positively activating *SOC1* (Figure 4H). An increase in SVP/FLC complex activity (as in *fve* or *fca* mutants) strongly delayed flowering under SDs and drought conditions, without affecting DE response under LDs (Figure 1C, 1D, 2A and 2B). Similarly *35S:SVP* plants did not exhibit DE response defects under LDs. These observations indicate that under LDs, GI–enabled, ABA–dependent florigen genes upregulation prevail over floral repression (Figure 5).

SOC1 Potentiates the Drought–Dependent *TSF* Upregulation

soc1 plants displayed strongly attenuated drought–dependent *TSF* upregulation (Figure 4D). Thus, the DE non–responsive phenotype of *soc1*

might derive from the combined effects of impaired *TSF* upregulation and defective signaling downstream of *FT*. Beyond flowering time control, *SOC1* is emerging as an important regulator of several developmental and stress responses. In conjunction with *FUL*, *SOC1* controls meristem determinacy and cambial activity (Melzer et al., 2008). Furthermore, *SOC1* orchestrates freezing tolerance responses by negatively regulating the *C–REPEAT / DRE–BINDING FACTOR* (*CBF*) genes (Seo et al., 2009). A genome–wide survey of *SOC1* binding sites revealed a significant enrichment in genes involved in abiotic stress responses process (Tao et al., 2012). The reduced *TSF* levels in *soc1* mutants coupled with the fact that drought–mediated *SOC1* upregulation was strictly ABA–dependent suggests a role for *SOC1* in mediating part of an ABA–dependent transcriptional control over *TSF*. We speculate that *SOC1* may also play a general role in coordinating other ABA–dependent responses.

The Phytohormone ABA Participates in the Floral Transition, but its Effect is Photoperiod Dependent

ABA levels increase upon water scarcity to orchestrate different drought responses (Leung and Giraudat, 1998; Nambara and Marion-Poll, 2005). However, ABA is regarded as a general inhibitor of flowering, as exogenous ABA applications delay flowering (Blazquez et al., 1998; Domagalska et al., 2010). Also, *glucose insensitive 1* (*gin1*, allelic to *aba2*) is early flowering compared with wild type Wassilewskija (Cheng et al., 2002; Domagalska et al., 2010). However, plants overexpressing the ABA biosynthesis rate–limiting enzyme *NCED3*, did not exhibit a significantly altered flowering phenotype (Domagalska et al., 2010). Recent findings suggest a positive role for ABA in stress–induced flowering by promoting the nuclear tethering of OXIDATIVE STRESS 2 (OXS2) zinc–finger transcription factor, an

activator of *SOC1* (Blanvillain et al., 2011). The late flowering phenotype we observed in independent ABA biosynthetic mutants (Columbia background) coupled with their reduced DE response also indicates that endogenous ABA acts as positive regulator of flowering under LDs. Supporting a positive role of ABA in flowering, constitutively activated ABA signaling mutants – e.g. *hab1–1 abi1–2 pp2ca–1*, (Rubio et al., 2009) – were early flowering under LDs. Also, the ectopic expression of the ABA–activated *Snrk2.6*/*OPEN STOMATA 1* (*OST1*) (a positive ABA signaling regulator) has been reported to produce an early flowering phenotype (Zheng et al., 2010).

Alongside these positive ABA effects on flowering (which could be explained in terms of patterns of *SOC1* and florigen activation) our data reveal a negative role of drought and ABA under SDs. Compared with wild type, *hab1–1 abi1–2 pp2ca–1* exhibited a late flowering phenotype under this photoperiod condition (Figure 3A). Also, in wild–type plants drought caused a floral delay compared with normal watering control and this was strongly dependent upon FLC/SVP complex activity (Figure 2A and 2C). However, *FLC* (but not SVP) transcript levels were only slightly up–regulated in wild–type plants upon drought conditions and in *hab1–1 abi1–2 pp2ca–1* under SDs (Figure 3G and Figure S4A and S4B). These data point to a model where in the absence of LDs, drought stress increases the repressor activity of the FLC/SVP complex largely at the post–transcriptional levels (Figure 5). It must be noted that drought treated wild–type plants under SDs did not phenocopy *hab1–1 abi1–2 pp2ca–1* mutants undergoing normal watering in terms of floral delay phenotype. These observations indicate that drought stress alone could not recapitulate the full effect of constitutive ABA signaling. Alternatively, the constitutive ABA

activation of *hab1–1 abi1–2 pp2ca–1* mutants could result in additional effects that were independent of ABA.

Different hormonal signals participate to the floral transitions by affecting florigen levels. GAs accelerates flowering through the upregulation of *FT* and *TSF* in the leaves (Galvão et al., 2012; Porri et al., 2012). Cytokinins (CKs) specifically activate *TSF* transcription (D'Aloia et al., 2011). However, the mode of action of GAs and CKs with respect to *FT* and *TSF* upregulation appears to be independent of the photoperiod conditions. Salicylic acid application also resulted in *FT* upregulation and early flowering (Martínez et al., 2004). Interestingly, this early flowering phenotype was dependent upon GI activity, but not CO, which is reminiscent of the DE response.

Expanding sets of gene expression data indicate a positive role for ABA and drought stress in the activation of florigen–like genes including *TSF*, *BROTHER OF FT AND TFL1* (*BFT*) and *MOTHER OF FT AND TFL1* (*MFT*) (Chung et al., 2010; Xi et al., 2010). In contrast, *ABA INSENSITIVE 3* (*ABI3*) has been proposed to negatively regulate *TSF* (Suzuki et al., 2003). Our data indicate an important role for ABA in the transcriptional upregulation of *FT* and *TSF*, but limitedly to long day photoperiod (Figure 4A and 4C). Moreover, increased *FT* levels (but not *TSF*) were observed in the *hab1–1 abi1–2 pp2ca–1* ABA hypersensitive mutants under LDs (Figure 3D). Thus, *TSF* requires both drought– and ABA–specific components for its upregulation. Indeed, besides the ABA–dependent activation of *TSF* we found evidence for an ABA–independent mechanism of activation, which could contribute to the residual DE response of *aba1* mutants (Figure 3A, 3B and 4C). Conversely, the late flowering phenotype of *hab1–1 abi1–2 pp2ca–1* mutants under SDs suggests also an inhibitory role for ABA in flowering. ABA is a mobile molecule and its site of production and

distribution are compatible with a role in the leaf vasculature (the site of florigen production) as well as the SAM (Endo et al., 2008; Seo and Koshiba, 2011). The opposing role of ABA in flowering may reflect spatially distinct ABA signaling mechanism (the leaf and the SAM). Thus, a more precise understanding of the site of ABA action as well as the mechanism for the ABA repressive role warrants further investigation.

CONCLUSIONS

Our data reveal an interaction between drought stress and photoperiod in the activation of the florigen genes, a process requiring photoperiod – activated GI protein and the phytohormone ABA. The ability to trigger a DE response allows plants to survive in ephemeral environments, characterized by sudden and unpredictable changes in water availability. As our data suggest the onset of the DE response to be tightly controlled by photoperiodic cues, drought episodes occurring in spring may be a cue for plants for yet harsher drought conditions to follow in the summertime, making a drought escape response advantageous. We propose that the broader significance for this photoperiod–drought stress interaction could be to allow water status signals to affect the floral transition, but limiting this to a particular temporal window (e.g. spring vs. autumn).

MATERIALS AND METHODS

Plant Materials and Growing Conditions

In this study we used wild–type *Arabidopsis* plants, ecotype Columbia (Col–0) or Landsberg *erecta* (L*er*). Mutant or transgenic lines (obtained from NASC or other laboratories) are detailed in the Table S1 online. Seeds were stratified in the dark at 4°C for 2 days before sowing. Seeds were germinated and plants grown in a controlled–environment cabinet at a temperature of 20 to 23 °C, 65% relative humidity, either under long day (16 h light / 8 h dark) or short day (8 h light / 16 h dark) photoperiods. Light was cool white fluorescent tubes (Osram, Sylvania) at a fluency of 120 to 150 $\mu mol\ m^{-2}\ s^{-1}$ (Photosynthetically active radiation).

Plants were grown in Arabasket pots plus Araflat (BETATECH, Gent, Belgium) filled with a blend (4:1, v:v) of loam sandy soil and peat (Vigorplant Italia). The soil water capacity was calculated as follows: Arabasket pots were filled with soil and air–dried for 72 h in an oven at 45 °C and then weighed (dry weight, DW). Arabasket pots were subsequently soaked in water and weighed (wet weight, WW). 100% RSWC (Relative Soil Water Content) was calculated with the following formula: (WW-DW)/(WW-DW) * 100. The water evaporation rate in the growth chambers was then calculated by air–drying the Arabasket pots and weighing them daily until the RSWC reached the target level of 30%. At least 15 plants were tested for each genotype in two parallel experiments: NW (80 – 90% RSWC) and LW conditions (30% RSWC). The RSWC was kept constant by daily application of 4 ml of water to the normally–watered and 2 ml every 2 days to the low watering plants. Throughout all the experiments, random Arabasket pots were weighed to monitor the RSCW. In all experiments plants received 2 ml of 1X solution of fertilizer every three weeks (NPK 7,5–3–6 + Fe, COMPO, Italy.).

For the SDs to LDs shift experiments, stratified seeds (20–50) were sown in Arabasket pots and plants grown as described above. After three weeks plants were harvested at the indicated time points of the subjective day and shifted to LDs. For each time point / treatment / genotype combination, plants were harvested in two biological replicates, each one consisting of approximately 50 seedlings pooled from three different Arabaskets. Two independent shift experiments were performed.

Isolation of Double Mutants and Genotyping

Mutant combinations were generated by crossing. The *agl24–2* and *svp–41* mutants alleles were genotyped as previously detailed (Michaels Scott D et al., 2003; Gregis et al., 2006). *gi–100* homozygous were selected using the BASTA resistance carried by the T–DNA. The *aba1–6* mutants were selected by genomic PCR amplification with primers flanking the *aba1–6*–specific polymorphism followed by BsaI restriction (Niyogi et al., 1998; Barrero et al., 2005). Genotyping primers for *soc1–2*, and *aba1–6* and RT–PCR primers for *fd–10*, *fkf1–10* and *ztl–10* are listed in the Table S2 online. *FD*, *FKF1* and *ZTL* transcript abundance in the *fd–10*, *fkf1–10* and *ztl–10* mutants was verified by RT–PCR (Figure S6).

Flowering Time Measurement and Quantification of DE

Flowering time was measured by counting the number of vegetative leaves produced at bolting. The drought escape response was calculated for each genotype as the percentage variation in the number of vegetative (rosette) leaves in plants grown under low watering condition (Leaves LW) relative to plants with a normal watering regime (Leaves NW) by the following formula: (Leaves LW- Leaves NW) / Leaves NW % Each mutant genotype / treatment combination experiment described in this work was repeated 2 to 4 times.

RNA Extraction and Real–time qPCR

Total RNA was extracted with TRIzol reagent (Invitrogen). A total of 1.5 µg of total RNA was used for cDNA synthesis with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real–time PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems), and amplification was real–time monitored on a 7900 HT Fast Real–time PCR system (Applied Biosystems). Changes in gene expression were calculated relative to *ACT2* using the ΔΔCt method (Livak and Schmittgen, 2001). Quantitative real–time PCR primers are provided in Table S2 online.

SUPPLEMENTAL MATERIAL

Figure S1. DE Response Induction in Arabidopsis.

Figure S2. Absence of DE Response in Independent *gi* Alleles.

Figure S3. DE Response in Floral Repressors Mutants Under SDs.

Figure S4. Floral Genes Regulation Under Drought Stress Upon SDs to LDs Shifts.

Figure S5. Mean Rosette Leaves Number in *aba1* mutants.

Figure S6. Characterization of T–DNA Insertion Alleles of *FD*, *FKF1* and *ZTL*.

Table S1. Flowering time of mutant and transgenic plants used in this study.

Table S2. List of Genotypes Used in this Study.

Table S3. List of Primers Used in this Study.

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Figure 1

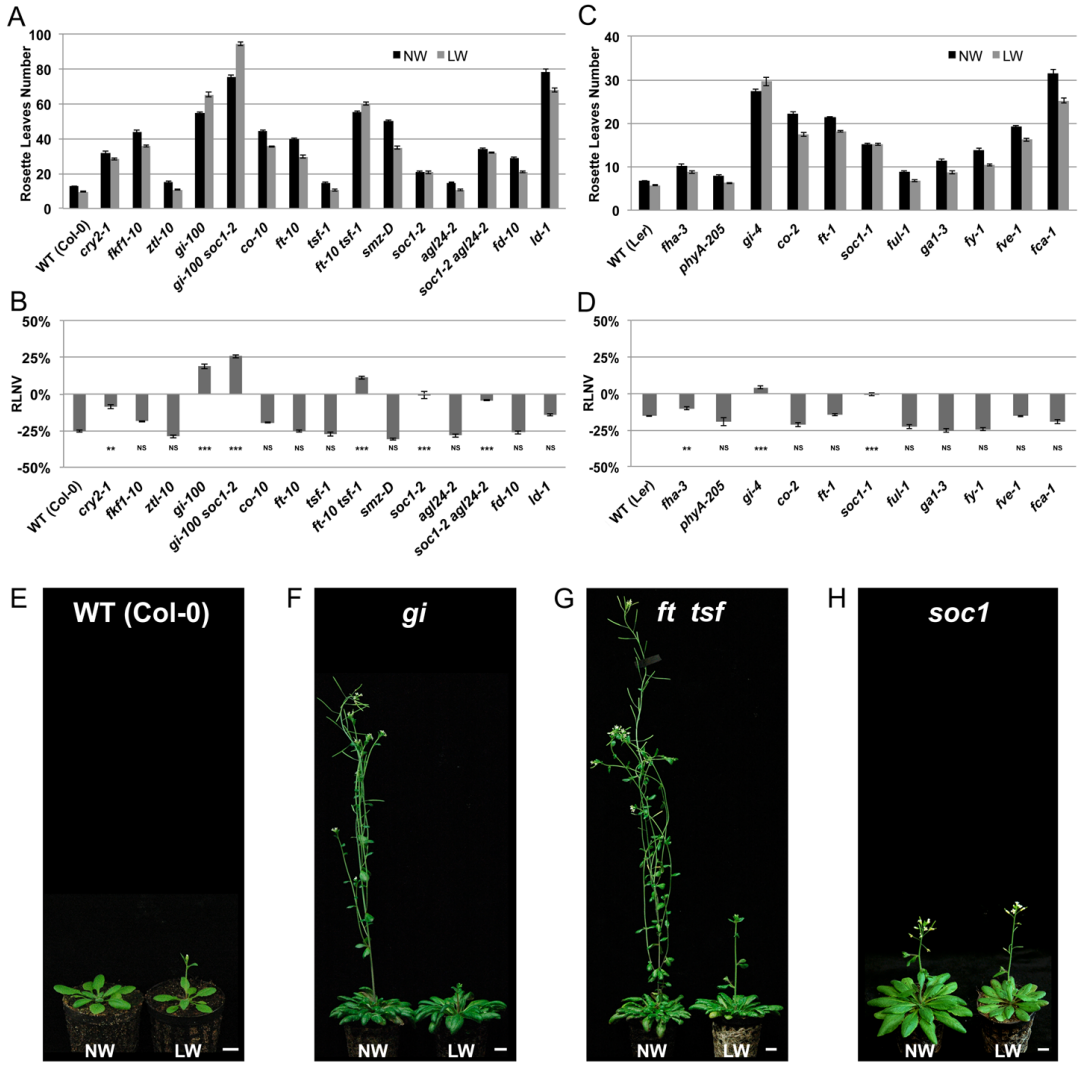

Figure 1. DE Response Requires Components of the Photoperiodic Pathway.

(**A**) and (**C**) Rosette leaves mean number of wild type (Col–0), (**A**), or L*er*, (**C**), and relative flowering time mutants grown under LDs. Plants were subjected to normal watering (NW, black bars) or low watering (LW, grey bars) regimes. Error bars represent ± SE n = 15.
(**B**) and (**D**) Quantification of DE response for each genotype detailed in (**A**) and (**C**), respectively, expressed as relative leaves number variation (RLNV). Numbers indicate percentage variations in number of leaves (%) in plants grown under LW condition relatively to NW. Error bars represent ± SE, Student's t test P ≤ 0.01 (**), ≤ 0.001 (***), > 0.05 not significant (NS).
(**E**) to (**H**) Images of representative plants of the indicated genotypes grown under LDs and subjected to NW or LW regimes. Wild–type Col–0 plants are 3 week–old (**E**), *gi–2* are 12 week–old (**F**), *ft–10 tsf–1* are 16 week–old (**G**) and *soc1–2* are 8 week–old (**H**). Scale bars = 1 cm

Figure 2

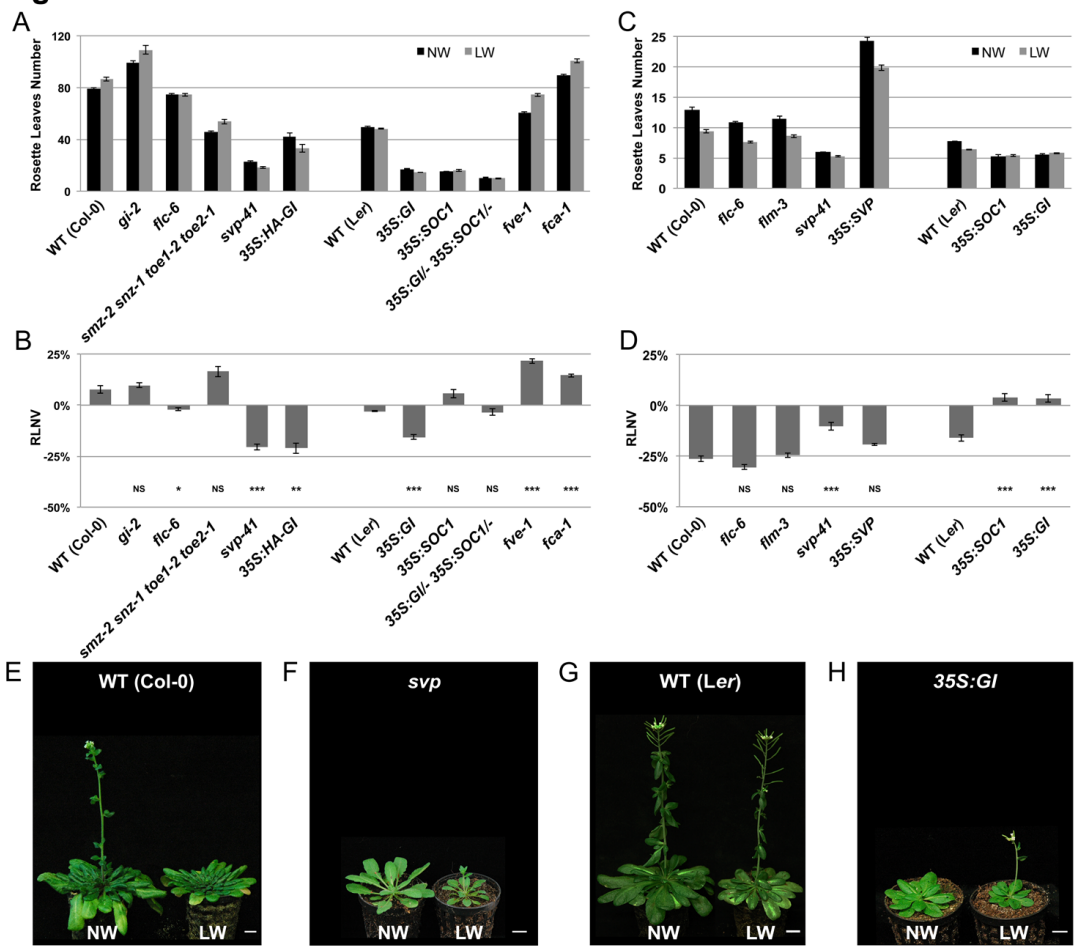

Figure 2. The Onset of DE Response is Photoperiod Dependent.

(**A**) Rosette leaves mean number of wild type plants and flowering time mutants grown under SDs. Plants were subjected to NW (black bars) or LW (grey bars) regimes. Error bars represent ± SE n = 15.
(**B**) Quantification of DE response for each genotype detailed in (**A**) expressed as RLNV. Error bars represent ± SE, Student's t test P values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***), > 0.05 not significant (NS).
(**C**) Rosette leaves mean number of wild type and flowering time mutants grown under LDs. Plants were subjected to NW (black bars) or LW (grey bars) regimes. Error bars represent ± SE n = 15.
(**D**) Quantification of DE response for each genotype detailed in (**C**) expressed as RLNV. Error bars represent ± SE, Student's t test P values ≤ 0.001 (***), > 0.05 not significant (NS).
(**E**) to (**H**) Images of representative plants of the indicated genotypes grown under SDs and subjected to NW or LW regimes. Wild–type Col–0 plants are 16 week–old (**E**), *svp–41* are 8 week–old (**F**), wild–type L*er* are 12 week–old (**G**) and *35S:GI* are 7 week–old (**H**). Scale bars = 1 cm.

Figure 3

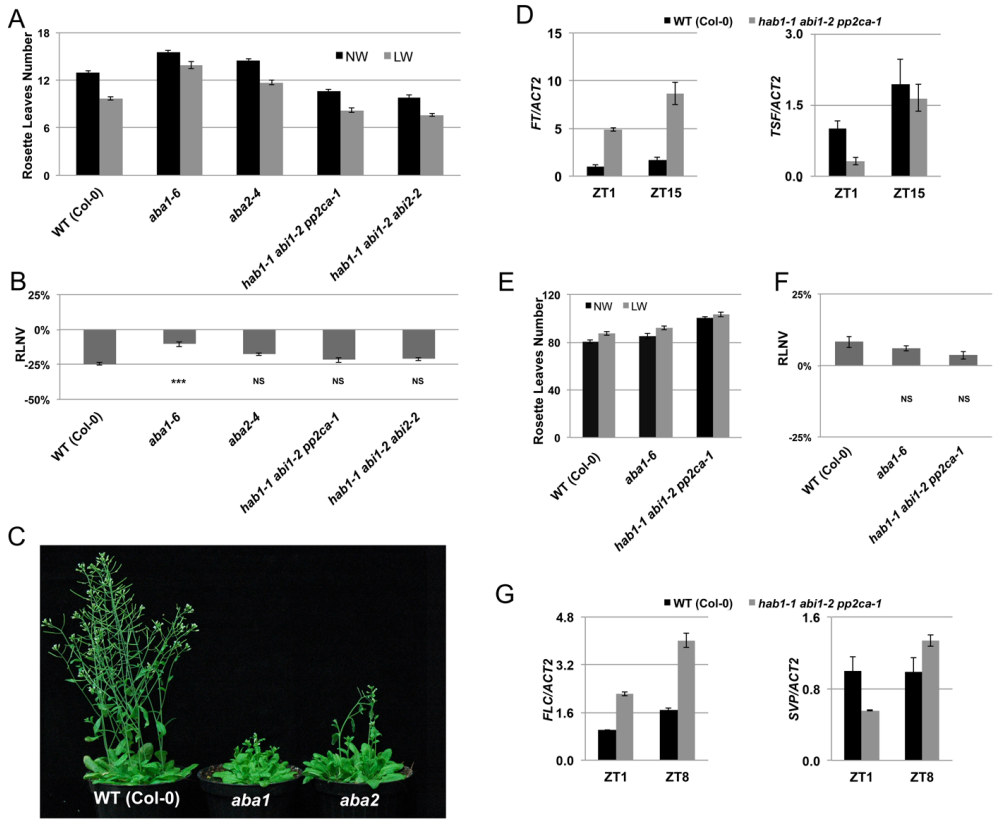

Figure 3. ABA is Required for DE Response by Positively Regulating Flowering.

(**A**) Rosette leaves mean number of wild type and ABA biosynthesis or signaling mutants grown under LDs. Plants were subjected to NW (black bars) or LW (grey bars) regimes. Error bars represent ± SE n = 15.
(**B**) Quantification of DE response for each genotype detailed in (**A**) expressed as RLNV. Error bars represent ± SE, Student's t test P values ≤ 0.001 (***), > 0.05 not significant (NS).
(**C**) Images of ABA biosynthesis deficient plants (*aba1–6* and *aba2–4*) compared with Col–0 wild type. 4 week–old plants grown under LDs are shown.
(**D**) Real–time qPCR of *FT* and *TSF* transcripts in 11 day–old *hab1–1 abi1–2 pp2ca–1* or wild–type (Col–0) seedlings. Plants were harvested at Zeitgeber 1 and 15 (ZT1, ZT15) in a 16 h light / 8 h dark photoperiodic regime. Values represent fold change variations of *FT* and *TSF* transcript levels relatively to ZT1 (arbitrarily set at 1 in Col–0). *ACTIN2* (*ACT2*) expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.
(**E**) Rosette leaves mean number of wild type and ABA biosynthesis or signaling mutants grown under SDs. Plants were subjected to NW (black bars) or LW (grey bars) regimes. Error bars represent ± SE n = 15.
(**F**) Quantification of DE response for each genotype detailed in (**E**) expressed as RLNV. Error bars represent ± SE, Student's t test P values > 0.05 not significant (NS).
(**G**) Real–time qPCR of *FLC* and *SVP* transcripts in 3 week–old *hab1–1 abi1–2 pp2ca–1* or wild–type (Col–0) seedlings. Plants were harvested at Zeitgeber 1 and 8 (ZT1, ZT8) in an 8 h light / 16 h dark photoperiodic regime. Values represent fold change variations of *FLC* and *SVP* transcript levels relatively to ZT1 (arbitrarily set at 1 in Col–0). *ACT2* expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.

Figure 4

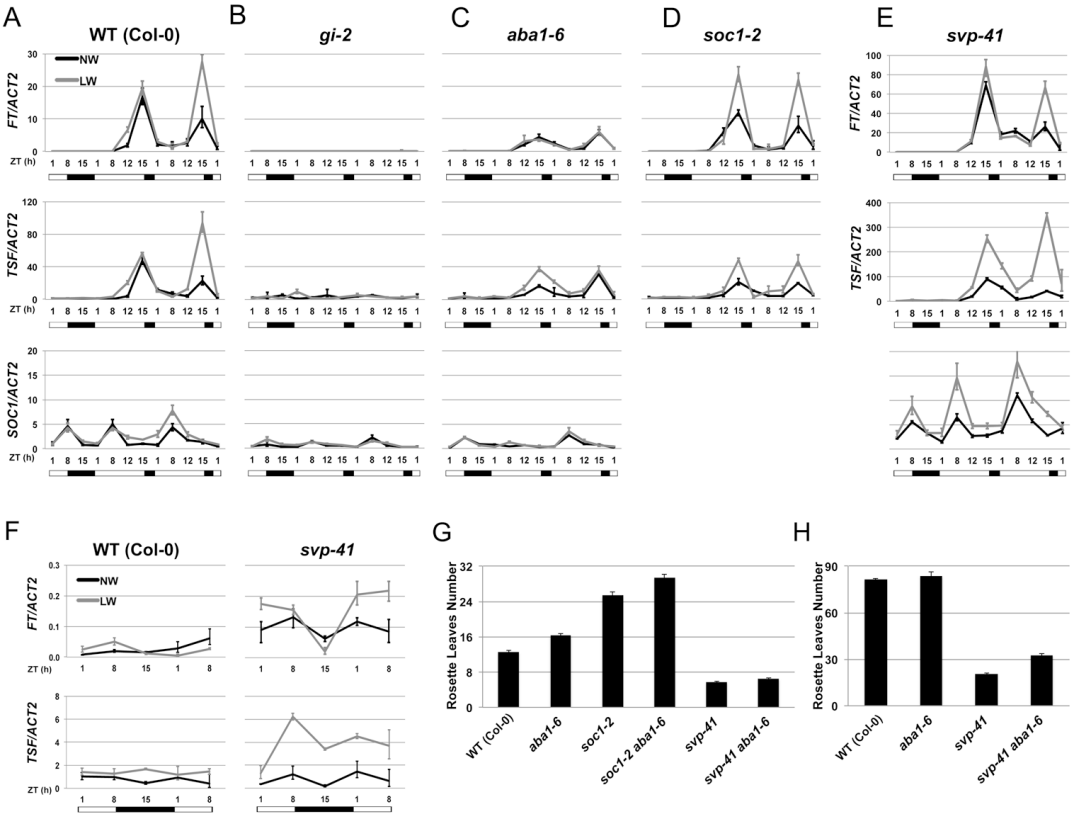

Figure 4. ABA– and Photoperiod– Dependent Upregulation of *FT*, *TSF* and *SOC1* Transcripts.

(**A**) to (**E**) Real–time qPCR of *FT*, *TSF* and *SOC1* transcripts in 3 week–old wild–type (Col–0) (**A**), *gi–2* (**B**), *aba1–6* (**C**), *soc1–2* (**D**) and *svp–41* (**E**) seedlings. Plants were subjected to NW (black lines) or LW (grey lines) regimes and harvested at the indicated time points in coincidence with the light phase (open bar) or in the dark (black bar) during a SDs to LDs shift. At each time point, values represent fold change variations of *FT*, *TSF* and *SOC1* transcript levels relatively to Col–0 under NW. *ACT2* expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.
(**F**) Close up of *FT* and *TSF* pattern of expression during the short day part of the experiment illustrated in (**A**) and (**E**).
(**G**) Rosette leaves mean number of wild–type (Col–0) and the indicated single and double mutants grown under LDs. Error bars represent ± SE n = 10 – 12.
(**H**) Same as (**G**) but grown under SDs. Error bars represent ± SE n = 10 – 12.

Figure 5

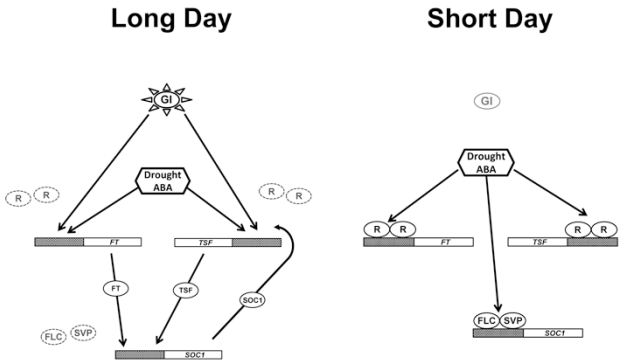

Figure 5. Photoperiod Dependency of the DE Response in Arabidopsis.

Drought stress, largely via ABA signaling promotes DE response under LDs but not SDs. Photoperiod–activated GI may relieve the transcriptional repression at the *FT/TSF* promoters, thus facilitating their ABA–dependent upregulation. Increased florigen levels trigger *SOC1* activation, which in turn contribute to *TSF* upregulation. A floral delay occurs under SDs upon drought conditions. Drought and/or ABA may enhance the activity of different repressor complexes (e.g. *SVP/FLC* or other repressors, R) through an unknown mechanism, thus interfering with the floral transition

Supplemental Data

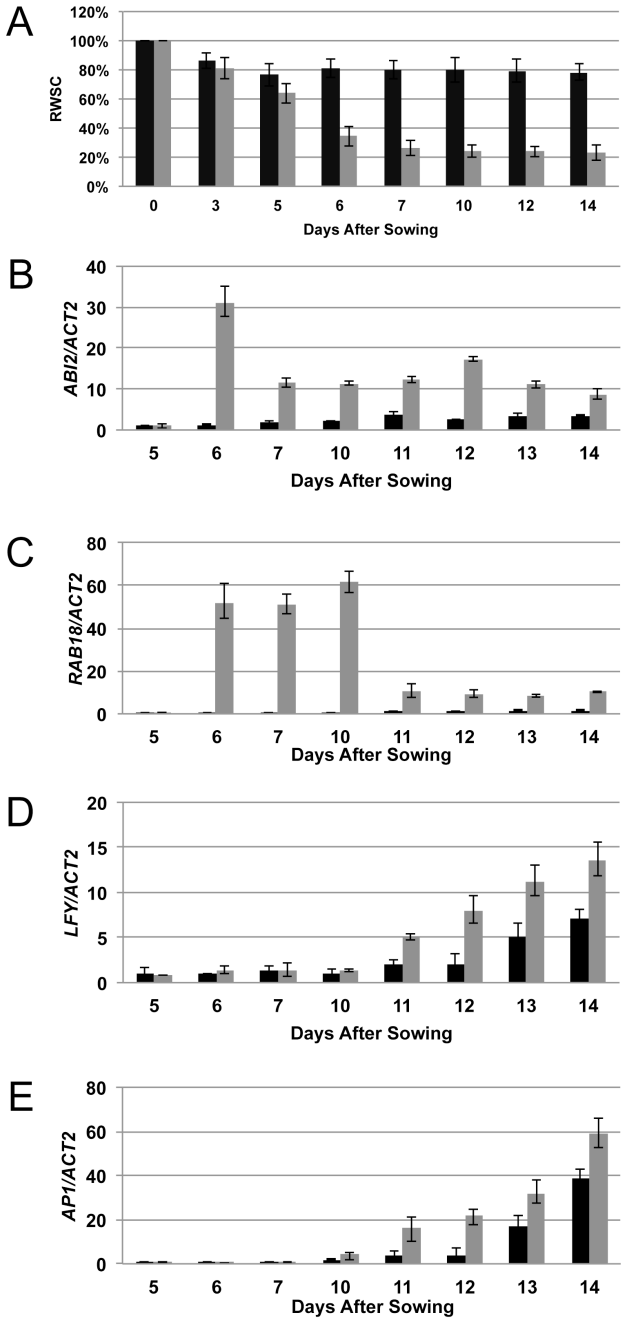

Figure S1. DE Response Induction in Arabidopsis.

(A) Mean relative soil water content (RSWC) values in Arabaskets pots of plants undergoing normal watering (NW, black bar) and low watering (LW, grey bar) treatments. Pots (n = 17) were weighed at the indicated days in 16 h light / 8 h dark photoperiod.

(B) to **(E)** Real-time qPCR transcript analyses of ABA and floral markers in wild-type (Col-0) plants grown under a 16 h light / 8 h dark photoperiod as detailed in (**A**). At least 20 seedlings from 3 different pots were harvested at the indicated days after sowing at Zeitgeber time 8. Values represent fold change variations of *ABI2* (**B**)*, RAB18* (**C**)*, LFY* (**D**) and *AP1* (**E**) transcript levels relatively to day 5. *ACTIN2* expression (*ACT2*) was used for normalization; error bars represent SE of two technical replicates. A representative experiment of two biological replicates is shown.

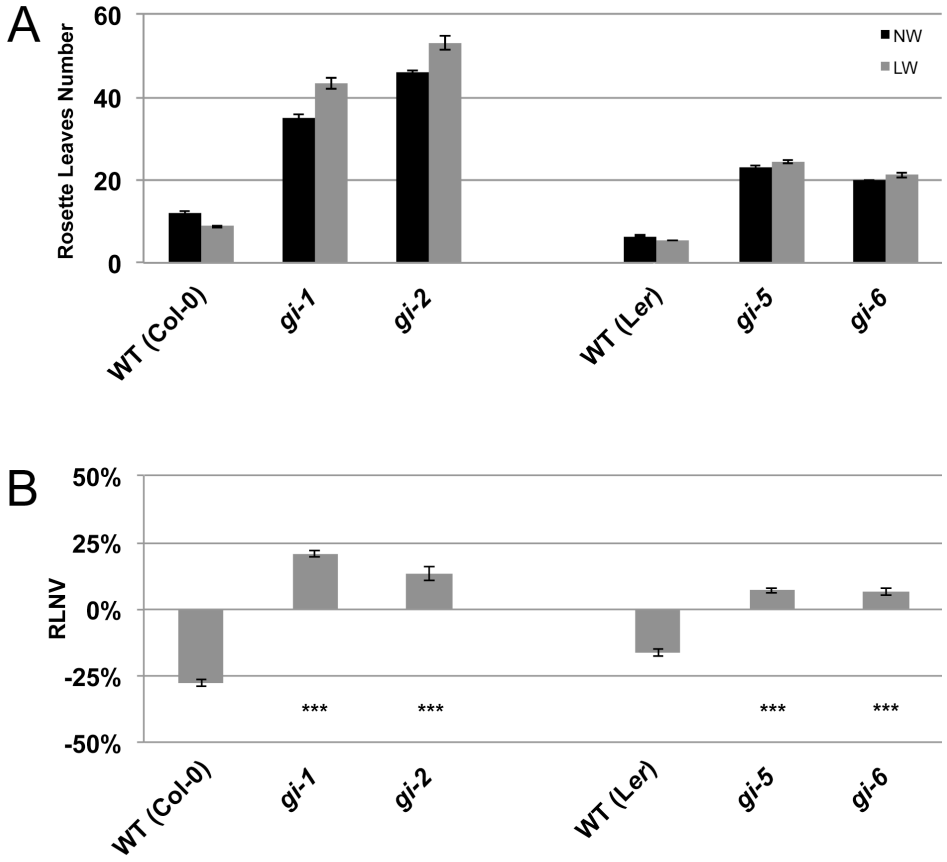

Figure S2. Absence of DE Response in Independent *gi* Alleles.

(A) Rosette leaves mean number of wild type and *gi* mutants grown under LDs. Plants were subjected to NW (black bars) or LW (grey bars) regimes. Error bars represent ± SE n = 15.

(B) Quantification of DE response for each genotype detailed in (**A**) expressed as relative leaves number variation (RLNV). Error bars represent ± SE, Student's t test P values ≤ 0.001 (***).

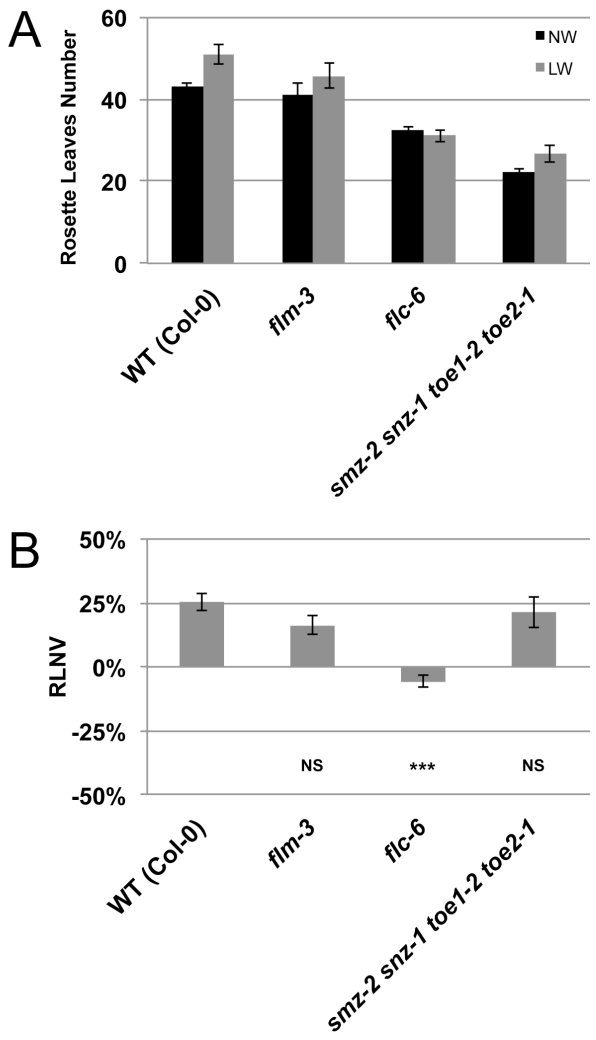

Figure S3. DE Response in Floral Repressors Mutants Under SDs.

(A) Rosette leaves mean number of wild-type and the indicated floral repressor mutants grown under SDs (10 h light / 14 h dark). Plants were subjected to NW (black bars) or LW (grey bars) regimes. Error bars represent ± SE n = 17.

(B) Quantification of DE response for each genotype detailed in (**A**) expressed as RLNV. Error bars represent ± SE, Student's t test P values ≤ 0.001 (***), > 0.05

not significant (NS).

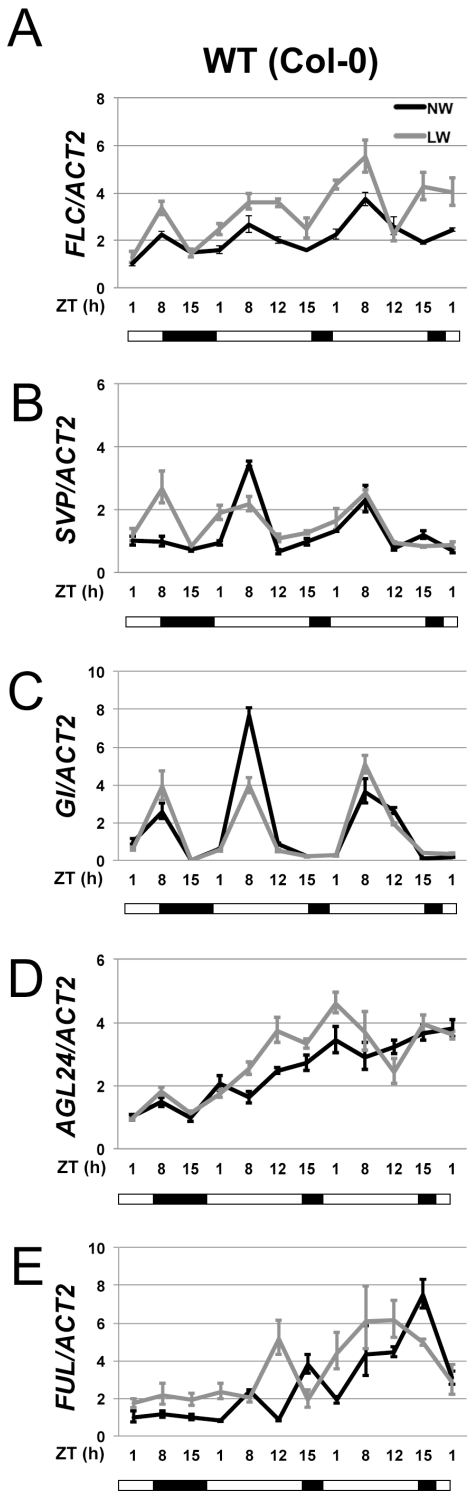
A
WT (Col-0)
NW
LW
FLC/ACT2
ZT (h)
1 8 15 1 8 12 15 1 8 12 15 1
B
SVP/ACT2
C
GI/ACT2
D
AGL24/ACT2
E
FUL/ACT2

Figure S4. **Floral Genes Regulation Under Drought Stress Upon SDs to LDs Shifts.**

(A) to **(E)** Real-time qPCR of floral genes transcripts in 3 week-old wild-type seedlings. Plants were subjected to NW (black lines) or LW (grey lines) regimes and harvested at the indicated time points (Zeitgeber, ZT) in coincidence with the light phase (open bar) or in the dark (black bar) during a SDs to LDs shift. At each time point, values represent fold change variations of transcript levels of *FLC* **(A)**, *SVP* **(B)**, *GI* **(C)**, *AGL24* **(D)** and *FUL* **(E)** relatively to the first ZT1 (arbitrarily set at 1 in normal watering control). *ACT2* expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.

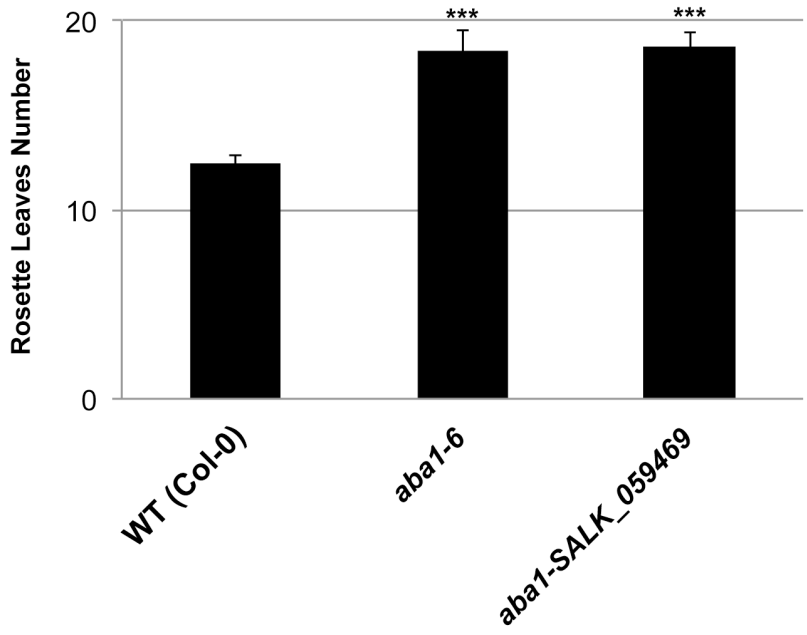

Figure S5. Mean Rosette Leaves Number in *aba1* mutants.

Rosette leaves mean number of wild-type and the indicated *aba1* alleles grown under LDs (16 h light / 8 h dark). Error bars represent ± SE n = 9 to 17. *** indicate Student's t test P values ≤ 0.001.

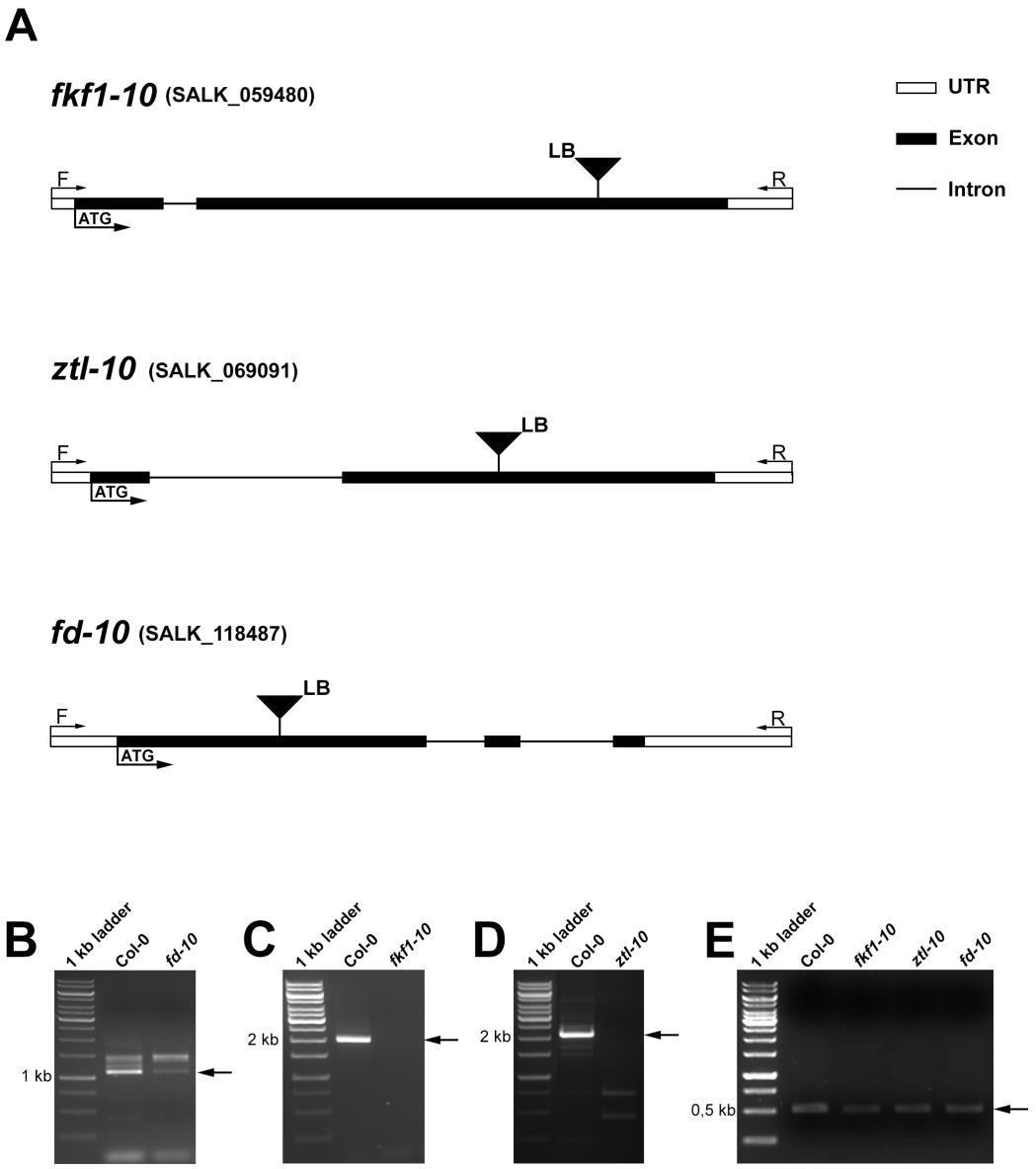

Figure S6. Characterization of T-DNA Insertion Alleles of *FD*, *FKF1* and *ZTL*.

(A) Diagram illustrating the T-DNA insertion alleles of *FD*, *FKF1* and *ZTL*. The positions of the forward (F) and reverse (R) primers used for RT-PCR and the T-DNA Left Board (LB) orientation are shown. **(B)** to **(E)** RT-PCR analysis from total cDNA derived from wild-type and the indicated mutant plants. Gene–specific primers illustrated in **(A)** were used to

amplify *FD* **(B)**, *FKF1* **(C)**, *ZTL* **(D)** and *ACT2* **(E)**. Arrows indicate the expected size band. PCR was conducted for 40 cycles except for *ACT2* (25 cycles). PCR products were visualized with Ethidium Bromide on an Agarose gel.

Table S1

Fig	Genotype	Normal Watering VL	SE	range	Low Watering VL	SE	range	P (NW vs. LW)
1 A	**WT (Col–0)**	**12.6**	±0.3	11–15	**9.4**	±0.3	8–12	< .0001
	cry2–1	**32.1**	±1.1	28–44	**28.5**	±0.6	23–31	= .009781
	fkf1–10	**44.0**	±0.8	41–49	**36.0**	±0.7	33–41	< .0001
	ztl–10	**15.1**	±0.4	13–19	**10.8**	±0.2	10–12	< .0001
	gi–100	**54.6**	±0.6	51–59	**65.4**	±1.3	56–72	< .0001
	gi–100 soc1–2	**75.3**	±1.2	67–84	**94.6**	±1.0	88–103	< .0001
	co–10	**44.6**	±0.5	41–49	**35.6**	±0.4	33–38	< .0001
	ft–10	**39.7**	±0.8	33–44	**29.7**	±0.7	23–34	< .0001
	tsf–1	**14.6**	±0.4	13–18	**10.5**	±0.5	8–14	< .0001
	ft–10 tsf–1	**55.2**	±0.8	51–62	**60.2**	±0.8	53–64	= .000228
	smz–D	**50.2**	±0.5	47–53	**34.8**	±0.9	29–43	< .0001
	soc1–2	**20.8**	±0.4	18–24	**20.8**	±0.9	17–26	= .992085
	agl24–2	**14.8**	±0.4	12–18	**10.6**	±0.4	9–13	< .0001
	soc1–2 agl24–2	**34.0**	±0.5	31–37	**32.0**	±0.4	30–35	= 0.014153
	fd–10	**28.9**	±0.6	25–34	**21.1**	±0.5	18–24	< .0001
	ld–1	**78.4**	±1.7	67–88	**68.0**	±1.4	60–75	= .000317
1 C	**WT (L.er)**	**6.7**	±0.1	6–7	**5.7**	±0.1	5–6	< .0001
	fha–3	**10.2**	±0.3	8–13	**8.7**	±0.3	7–11	= .007010
	phyA–205	**7.8**	±0.3	6–10	**6.3**	±0.2	5–8	= .001191
	gi–4	**27.4**	±0.5	24–31	**29.7**	±1.0	26–32	= .046167
	co–2	**22.2**	±0.5	18–26	**17.5**	±0.5	12–22	< .0001

	ft–1	**21.3**	±0.3	19–25	**18.2**	±0.2	16–20	< .0001
	soc1–1	**15.2**	±0.2	14–17	**15.1**	±0.3	13–17	= .866151
	ful–1	**8.8**	±0.2	8–10	**6.7**	±0.2	6–8	< .0001
	ga1–3	**11.4**	±0.4	9–14	**8.7**	±0.3	7–11	< .0001
	fy–1	**13.8**	±0.5	12–17	**10.4**	±0.3	9–12	< .0001
	fve–1	**19.2**	±0.4	17–22	**16.2**	±0.4	14–19	< .0001
	fca–1	**31.4**	±1.0	23–44	**25.2**	±0.5	20–28	= .001037
2 A	**WT (Col–0)**	**79.5**	±0.8	77–87	**86.7**	±1.7	79–97	= .003456
	gi–2	**99.2**	±1.9	81–111	**108.9**	±3.4	88–130	= .0021275
	flc–6	**74.6**	±1.0	68–81	**74.5**	±1.0	69–77	= .952723
	smz–2 snz–1 toe1–2 toe2–1	**45.5**	±0.6	41–49	**53.6**	±1.5	48–59	< .0001
	svp–41	**22.9**	±0.6	19–27	**18.3**	±0.6	13–22	< .0001
	35S:HA–GI	**42.2**	±2.6	28–51	**32.9**	±2.9	18–41	= .020842
	WT (L*er*)	**49.6**	±0.6	44–52	**48.2**	±0.5	44–52	= .063810
	35S:GI	**17.1**	±0.3	15–19	**14.5**	±0.3	13–16	< .0001
	35S:SOC1	**15.1**	±0.5	13–19	**16.1**	±0.8	12–23	= .288215
	35S:GI/- 35S:SOC1/-	**10.4**	±0.4	8–14	**9.9**	±0.4	7–13	= .297334
	fve–1	**60.6**	±0.8	54–65	**74.5**	±1.4	69–84	< .0001
	fca–1	**89.6**	±0.9	85–95	**100.8**	±1.5	86–106	= .000309
2 B	**WT (Col–0)**	**12.9**	±0.5	10–16	**9.4**	±0.3	7–12	< .0001
	flc–6	**10.9**	±0.1	10–11	**7.6**	±0.2	7–9	< .0001
	flm–3	**11.5**	±0.4	8–14	**8.6**	±0.3	7–10	< .0001
	svp–41	**5.9**	±0.1	5–7	**5.3**	±0.1	5–6	= .000327
	35S:SVP	**24.3**	±0.5	22–28	**19.9**	±0.5	18–23	< .0001
	WT (L.er)	**7.7**	±0.1	6–7	**6.4**	±0.1	5–6	= .000353
	35S:SOC1	**5.3**	±0.2	4–6	**5.4**	±0.2	4–6	= .500149
	35S:GI	**5.5**	±0.1	5–6	**5.8**	±0.1	5–6	= .170316
3 A	**WT (Col–0)**	**12.9**	±0.3	11–15	**9.7**	±0.2	9–11	< .0001
	aba1–6	**15.5**	±0.3	13–17	**13.9**	±0.5	11–17	= .021215
	aba2–4	**14.5**	±0.3	13–16	**11.7**	±0.3	10–14	< .0001

	hab1–1 abi1–2 pp2ca–1	**10.7**	±0.2	10–11	**8.2**	±0.3	7–9	< .0001
	hab1–1 abi1–2 abi2–2	**9.8**	±0.4	6–15	**7.6**	±0.2	6–10	< .0001
3 E	**WT (Col–0)**	**80.5**	±1.3	62–84	**87.5**	±1.7	81–103	= .002435
	aba1–6	**85.3**	±2.3	71–103	**92.0**	±1.6	81–99	= .108272
	hab1–1 abi1–2 pp2ca–1	**100.5**	±1.3	88–109	**103.4**	±2.0	95–119	= .270123

Fig	Genotype	VL	SE	range	P	vs.
4 G	**WT (Col–0)**	**12.5**	±0.4	10–16		
	aba1–6	**16.4**	±0.3	15–18	< .0001	Col
	soc1–2	**25.4**	±0.8	23–29	< .0001	Col
	soc1–2 aba1–6	**29.5**	±0.7	26–33	= .00057	*soc1–2*
	svp–41	**5.7**	±0.2	5–6	< .0001	Col
	svp–41 aba1–6	**6.5**	±0.2	5–8	= 0.024885	*svp–41*
4 H	**WT (Col-0)**	**81.5**	±0.9	77–87		
	aba1–6	**83.8**	±2.6	71–99	= .102985	Col
	svp–41	**20.5**	±0.8	16–26		
	svp–41 aba1–6	**32.6**	±1.2	25–40	< .0001	*svp–41*

		Normal Watering			Low Watering			
Fig	Genotype	VL	SE	range	VL	SE	range	P (NW vs. LW)
SUP 2	**WT (Col-0)**	**12.2**	±0.4	10–15	**8.8**	±0.3	7–11	< .0001
	gi–1	**35.1**	±1.0	29–41	**43.4**	±1.5	32–58	< .0001
	gi–2	**46.1**	±0.6	43–50	**53.1**	±1.6	44–65	= .000208
	WT (L*er*)	**6.1**	±0.1	6–7	**5.5**	±0.1	5–6	< .0001
	gi–5	**23.1**	±0.5	21–27	**24.4**	±0.4	21–27	= .026375
	gi–6	**19.8**	±0.3	18–22	**21.1**	±0.5	18–26	= .025683

SUP 3	**WT (Col-0)**	**43.0**	±0.9	38–52	**50.9**	±2.4	33–69	= .008682
	flm-3	**41.0**	±3.1	22–59	**45.7**	±3.0	34–64	= .024636
	flc-6	**32,.4**	±0.8	27–39	**31.1**	±1.4	24–43	= .412885
	smz-2 snz-1 toe1-2 toe2-1	**22.2**	±0.8	20–25	**26.7**	±2.0	17–39	= .012685

Fig	Genotype	VL	SE	range	P	vs.
SUP 5	**WT (Col-0)**	**12.4**	±0.5	10–14		
	aba1–6	**18.4**	±1.1	14–23	< .0001	Col
	aba1-SALK_059469	**18.6**	±0.8	14–25	< .0001	Col

Table S1. Flowering time of mutant and transgenic plants used in this study.

Mean values of vegetative leaves (VL) and standard error (SE) of plants undergoing normal watering and low watering conditions. Two tailed Student's *t* test values (*P*) are shown.

Table S2

Allele	Background	Reference
ld–1	Col–1	(Lee et al., 1994)
fca–1	L*er*	(Koornneef et al., 1991)
fve–1	L*er*	(Koornneef et al., 1991)
fy–1	L*er*	(Koornneef et al., 1991)
cry2–1	Col–4	(Guo et al., 1999)
fha–3	L*er*	(Koornneef et al., 1991)
fkf–10	Col–0	This work
ztl–10	Col–0	This work
gi–100	Col–0	(Huq et al., 2000)
gi–1	Col–1	(Fowler et al., 1999)
gi–2	Col–1	(Fowler et al., 1999)
gi–4	L*er*	(Fowler et al., 1999)
gi–5	L*er*	(Fowler et al., 1999)
gi–6	L*er*	(Fowler et al., 1999)
35S:GI gi–3	L*er*	(Mizoguchi et al., 2005)
35S:HA–GI gi–2	Col–0	(David et al., 2006)
phyA–205	L*er*	(Reed et al., 1994)
co–2	L*er*	(Koornneef et al., 1991)
co–10	Col–0	(Laubinger et al., 2006)
ft–1	L*er*	(Koornneef et al., 1991)
ft–10	Col–0	(Yoo et al., 2005)
tsf–1	Col–0	(Yamaguchi et al., 2005)
ft–10 tsf–1	Col–0	(Jang et al., 2009)
fd–10	Col–0	This work
soc1–2	Col–0	(Lee et al., 2000)
soc1–1	L*er*	(Onouchi et al., 2000)
gi–100 soc1–2	Col–0	This work
35S:SOC1	L*er*	Samach et al. 2000
35S:GI -/35S:SOC1 -	Col–0	This work
agl24–2	Col–0	(Michaels Scott D et al., 2003)
soc1–2 agl24–2	Col–0	This work
ful–1	L*er*	(Gu et al., 1998)
svp–41	Col–0	(Hartmann et al., 2000)
35S:SVP	Col–0	(Gregis et al., 2009)
flc–6	Col–0	(Bouveret, 2006)
flm–3	Col–0	(Bouveret, 2006)

smz–2 snz–1 toe1–2 toe2–1	Col–0	(Mathieu et al., 2009)
smz–D	Col–0	(Mathieu et al., 2009)
***aba1*–SALK_059469**	Col–0	(Morris et al., 2006)
aba1–6	Col–0	(Niyogi et al., 1998; Barrero et al., 2005)
aba2–4	Col–0	(Laby et al., 2001)
hab1–1 abi1–2 pp2ca–1	Col–0	(Rubio et al., 2009)
hab1–1 abi1–2 abi2–2	Col–0	(Rubio et al., 2009)

Table S3

Gene	Forward	Reverse	Use
ACT	CTCTCCCGCTATGTATGTCGCCA	GTGAGACACACCATCACCAG	**qPCR**
GI	AATTCAGCACGCGCCTATTG	GTTGCTTCTGCTGCAGGAACTT	**qPCR**
FT	CTAGCAACCCTCACCTCCGAGAATA	CTGCCAAGCTGTCGAAACAATATAA	**qPCR**
TSF	CTCGGGAATTCATCGTATTG	CCCTCTGGCAGTTGAAGTAA	**qPCR**
SOC1	ATCGAGGAGCTGCAACAGAT	GCTACTCTCTTCATCACCTCTTCC	**qPCR**
FLC	TGTGGATAGCAAGCTTGTGG	TAGTCACGGAGAGGGCAGTC	**qPCR**
SVP	CCGGAAAACTGTTCGAGTTC	TGACTGCAAGTTATGCCTCTCT	**qPCR**
AGL24	GAGGCTTTGGAGACAGAGTCGGTGA	AGATGGAAGCCCAAGCTTCAGGGAA	**qPCR**
FD	GCTCACTTGCAGGCAGAAAA	CCTTTTCTCTTTCCGGGTCT	**qPCR**
FUL	TTGCAAGATCACAACAATTCGCTTCT	GAGAGTTTGGTTCCGTCAACGACGAT	**qPCR**
LFY	ACGTGGCAAAAAGAACGGCTTAGA	CGCGTACCTGAATACTTGGTTCGTC	**qPCR**
AP1	AGGGAAAAAATTCTTAGGGCTCAACAG	GCGGCGAAGCAGCCAAGGTTGCAGTTG	**qPCR**
RAB18	ATGGCGTCTTACCAGAACCGT	CCAGATCCGGAGCGGTGAAGC	**qPCR**
ABI2	GGAGTGACTTCGATTTGTGGTAGACG	GTCAAAGCCAGATGCATCCTCTCACG	**qPCR**
ZTL	CCACTCGTTTCTTTGTCCACC	TGAACACAAATGCACTTCTCAA	**RT-PCR**
FKF1	AGGCTGAGAGCTTATCAGAGA	TGTACACACGCTTCTAGCTTCT	**RT-PCR**
FD	AGCTGTGTTGTTGGTTCACT	GACAGGTGTTCCTGTGCCTT	**RT-PCR**
ACT	GTGTTGGACTCTGGAGATGGTGTG	GCCAAAGCAGTGATCTCTTTGCTC	**RT-PCR**
SOC1	ACTAAAGAAGAAGATATGGTGAGG	ATATCACAAACCGTTTAGAAGCTTCGAGTTGTTCA	**genotype**
soc1-2	TGGTTCACGTAGTGGGCCATCG	ATATCACAAACCGTTTAGAAGCTTCGAGTTGTTCA	**genotype**
aba1–6	GCTCGGAGTAAAGGCGGCGA	CAGGAAGTCCCCGTGACGCC	**genotype**

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Part III

Unpublished Data – Role of ABA in flowering

1 Exogenous ABA does not mimic the drought stress–mediated floral acceleration

My work revealed a positive role for the phytohormone ABA in affecting flowering time. However previous data in the literature suggest ABA as a negative regulator of the floral transition. (Barrero et al., 2005) showed that ABA biosynthetic mutants (*aba1-1*) are early flowering compared with wild type when grown *in vitro*. Other reports indicate that ABA applications on whole plants cause late flowering, which is in contrast with the effect of drought on soil – grown plants, which accelerates flowering.

To clarify these discrepancies I have grown wild type, ABA deficient (*aba1–1*) and ABA insensitive (*abi1–1*) plants under LDs conditions in phytatray containing growth medium supplied with ABA at different concentrations (previously shown to have an effect on flowering). ABA caused a similar delay in flowering in wild type and mutant plants, although increasing concentration did not aggravate this phenotype (Fig. 1A). As *abi1–1* mutants are impaired in the main mechanism of ABA signalling (Fig. 1A), the observed ABA– mediated delay in flowering is unlikely to involve the canonical ABA signalling pathway.

In the absence of ABA *aba1–1* was slightly early flowering compared with wild type but this phenotype could not be completely rescued by ABA. When grown on soil *aba1* did not display obvious defects in flowering time, but consistently showed a trend ($p \leq 0.082$) towards a late flowering phenotype (Fig 1B). However an independent *aba1* allele (*aba1–3*) was late flowering compared with WT (Fig. 6A). This is consistent with the phenotype of the *aba1-6* and *aba1-SALK_059469* mutants (Columbia background) (Fig 2A, B) thus excluding an allele dependency for the positive role of ABA in the floral transition. Taken together, these data may

suggest a non–physiological effect of growth medium on flowering time in mutants impaired in ABA production.

On soil, the combination of LDs and low watering conditions triggers the DE response mainly thought an ABA–dependent mechanism (Fig 1C, D; 3A, B Part II). I therefore sought to study the effects of exogenous ABA on soil–grown plants. Daily applications of ABA 100 µM significantly ($p < 0.01$) delayed flowering, irrespective of the watering status of the soil (Fig 1C). However, this treatment did not affect the ability of plants to accelerate flowering under drought conditions, thus resulting in a normal DE response (Fig 1D). This suggests that the ABA treatment affects the floral transition at different levels, independently of the physiological action of the drought signal.

The DE response is triggered by the transcriptional upregulation of the florigen genes and *SOC1* in a photoperiod– and ABA–dependent manner (Fig 4 – Part II). Intriguingly, publicly–available microarrays indicate that *SOC1* is upregulated upon ABA treatments (NASCARRAYS-176, http://affymetrix.arabidopsis.info). This raises the question as to why is exogenous ABA not resulting into early flowering despite the upregulation of *SOC1*. We monitored the expression of flowering genes in wild type and *abi1–1* plants at different time points after a shift to ABA–containing plates. Consistent with previous observations, *SOC1* was strongly upregulated upon an ABA shift in an ABI1–dependent manner (Fig. 1E, F). However, ABA had no effect on florigen upregulation. Rather, ABA caused a small downregulation of *FT* in WT and *abi1–1* plants (Fig 1E, F). Furthermore, ABA caused an upregulation of the floral repressors *FLC* and *SVP* (Fig. 1E, F). Interestingly *abi1–1* mutant showed a lower *FLC* expression level compared to WT under control conditions, but this was not reflected in altered *FT* levels. Collectively these results indicate that ABA applications

do not recapitulate the full range of transcriptional responses accompanying DE under drought conditions.

2 ABA interacts with photoperiod to modulate flowering

In the paper we proposed a model where photoperiod–activated GI allows ABA to positively upregulates *FT/TSF* and *SOC1* genes (Fig 5 – Part II). In support of this model, *aba1 soc1* double mutants were later flowering than their respective single mutants, indicating that ABA accelerates flowering through pathways other than *SOC1* (Fig 4 – Part II), for example *FT* and *TSF*. To investigate such hypothesis we generated double *ft–10 aba1–6* and triple *ft–10 tsf–1 aba1–6* mutants plants. *ft–10 aba1–6* plants were later flowering than *ft–10* (Fig. 2A, C). Similarly, *ft–10 tsf–1 aba1–6* were later flowering than double *ft–10 tsf–1* plants (Fig. 2A, D) although the effect of the *aba1–6* allele in the *ft–10 tsf–1* genetic background was somewhat attenuated compared to that in the single *ft–10* background. Collectively these data indicate that ABA plays a positive role in the floral transition largely (but not exclusively) through the action of *FT*/*TSF*. The fact *ft–10 tsf–1 aba1–6* was still later flowering than *ft–10 tsf–1* indicates the existence of a residual ABA–dependent pathway promoting the floral transition.

The observation that *aba* mutants under SDs conditions flowered as late as wild type suggests that ABA necessitates LDs (or photoperiod–activated GI) to trigger flowering (Fig. 3E – Part II). Corroborating this hypothesis, *gi–2 aba1–6* displayed a similar flowering phenotype compared to *gi–2* single mutants (Fig. 3A, E). Crucially, *gi–2 aba1–6* plants were similar to the *ft–10 tsf–1 aba1–6* in terms of flowering time indicating that GI influence the

whole ABA–dependent activation of the floral transition, which is still present in the *ft–10 tsf–1* background (Fig. 3A). This could include a florigen–independent pathway of *SOC1* activation or the activation of *MFT* (a third florigen protein). Evidences for a strong interplay between ABA and GI in activating florigen–like protein were indeed obtained. *BFT*, a gene with opposite function to *FT*, was strongly upregulated under drought stress, in particular during the LD part of the experiment (Fig 3F). This upregulation was abolished in *aba1–6* and *gi–2* mutants (Fig 3G, H). Thus, GI might regulate the transcriptional balance of positive (*FT*, *TSF* and *MFT*) and negative (*TFL*, *BFT* and *ATC*) florigens by allowing their ABA–dependent upregulation.

The intimate relationship between ABA and photoperiod was also apparent in mutants with constitutively ABA signalling (*hab1–1 abi1–2 pp2ca–1)*. Compared with wild type, these plants flowered early under LDs (Fig 3A – Part II). *FT* levels were higher in these plants, but limitedly to LDs conditions, suggesting that *FT* transcription is positively targeted by ABA (Fig. 3D – Part II). We performed further analysis on *hab1–1 abi1–2 pp2ca–1* mutants, this time at several time points (comprising SDs and LDs) and comparing different watering regimes. *FT* transcript levels in *hab1–1 abi1–2 pp2ca–1* plants were similar to wild type during the SD part of the experiment (Fig. 3A, B). This is consistent with the notion that elevated ABA signalling cannot override the LD photoperiod requirement for *FT* activation. However, upon a LD shift, *hab1–1 abi1–2 pp2ca–1* mutants displayed an increased *FT* upregulation compared with wild type, both under normal watering or drought conditions (Fig. 3A, B). Unlike *FT*, *TSF* transcript levels were similar in both genotypes (Fig. 3A, B), suggesting that *FT* is the main target of ABA signalling in the contest of flowering time control. *SOC1* levels did not greatly vary in *hab1–1 abi1–2 pp2ca–1* compared to wild type

plants under SDs conditions, irrespective of the watering regimes. However, a stronger *SOC1* upregulation occurred upon a LD shift in the *hab1–1 abi1–2 pp2ca–1* compared with wild type, both under normal or low watering conditions (Fig. 3A, B), which could be interpreted as a consequence of more *FT* being present, and/or as a direct action of ABA on *SOC1*.

Under SDs the *hab1–1 abi1–2 pp2ca–1* mutants are later flowering compared to wild type (Fig. 3 – Part II) but the mechanism by which ABA signalling delays flowering under SDs is unknown. SPLs factors are important floral promoters under SDs, conveying plant age cues upon *SOC1* promoter (Wang et al., 2009) and GAs signal into the floral transition mechanisms (Yamaguchi et al., 2009; Porri et al., 2012). The analysis of four independent *SPL* genes in plants undergoing drought stress did not reveal any major decrease in their expression (Fig. 4). In contrast, all four *SPL*s were slightly upregulated in the SD part of the experiment under drought conditions compared to normal watering (Fig. 4). These results suggest that ABA delays the floral transition under SDs downstream of the *SPL*s. Because we found no evidence for altered *SOC1* levels in SDs under drought conditions (Fig. 3A), ABA might interfere with SOC1 activity in the SAM.

The analysis of *SVP* and *FLC*, two negative regulators of the DE response, in *hab1–1 abi1–2 pp2ca–1* may further inform about the negative role of ABA on flowering under SDs. The pattern of *SVP* transcript accumulation was similar in wild type and *hab1–1 abi1–2 pp2ca–1* plants at any time point or watering regime (Fig 3A, B). Unlike previously observed, the levels of *SVP* transcript appeared to be more dynamic and responsive to drought (although the scale of such variations is limited to maximum of 2 fold changes compared to normal watering). To test whether such

transcriptional changes were reflected in increased SVP protein accumulation we monitored the levels of a functional SVP:GFP fusion protein in transgenic *SVP*$_{pro}$*SVP:GFP* plants subjected to different watering and photoperiod regimes (Fig. 5). As expected SVP:GFP protein levels displayed diurnal changes, consistent with *SVP* transcript accumulation being under the control of the circadian clock. However, despite an increase in *SVP* transcript levels as a result of drought stress, no corresponding change in SVP:GFP protein levels was observed. Whether drought stress can affect SVP protein activity (e.g. by prompting different SVP post–translational modifications, protein–protein interactions, and/or chromatin occupancy) awaits further investigation.

In good agreements with previous findings, we found a general increase in *FLC* transcript levels in drought treated wild–type plants compared with normal watering (Fig. 4A). *FLC* levels were however strongly increased in *hab1–1 abi1–2 pp2ca–1* mutants compared to wild type, especially under drought conditions (Fig. 4A, B). As our genetic data point to an involvement of *FLC* and *SVP* in preventing DE response under SDs, this finding further suggests that ABA (alone and/or in combination with drought stress) promotes *FLC* transcription, thus contributing to delaying flowering under SDs.

3 Different components of the ABA signalling pathway distinctly affect flowering and DE response

Several components of the ABA signalling pathway mutants are known to display altered flowering time, especially under SDs conditions (Martínez-Zapater et al., 1994). We therefore aimed to study these mutants and characterise their DE phenotype.

The dominant ABA insensitive *abi1–1* and *abi2–1* mutants carry synonymous amino acid substitutions alleles that disrupt early ABA signalling at the ABA receptors level. Unlike the *aba1–3* (unable to produce ABA) *abi1–1* and *abi2–1* do not show clear flowering defects under LDs and normal watering conditions (Fig. 6A). However, under drought conditions *abi1–1* generated a significant floral delay, which was never observed in any *aba* mutant allele (Fig. 6B). Furthermore, *abi1–1* and *abi2–1* mutants were early flowering under SDs conditions (Fig. 6C), which is again at odds with the *aba* mutants.

ABI3 encodes a B1/B3 transcription factor responsible for the positive and negative regulation of a plethora of ABA responsive genes. *abi3* loss of function mutants are early flowering under both LDs and SDs conditions (Fig. 6A, B). We recently demonstrate that the *abi3–5* mutants display a dramatic increase in *FT* compared to wild type. This result may suggest that ABI3 is involved in the repression of the florigens.

While these initial data may contribute to shedding some light on the complex facet of ABA signalling in the control of the floral transition, it is still unclear how these different ABA components genetically interact (e.g. temporally and spatially) and at what levels are they regulated (e.g. transcriptionally or post–transcriptionally).

4 GI participates in the regulation of ABA signalling

ABA regulates the floral transition in a photoperiodic–dependent manner. We thus wondered how general this interconnection between photoperiod and ABA signalling was. The transcriptional analysis of well–characterised ABA markers during SDs to LDs shift experiments could provide useful insights on the effect of the day length on ABA signalling.

Under normal watering conditions, all tested ABA marker genes showed diurnal changes in transcript accumulation, consistent with ABA signalling being under the control of the circadian clock (Legnaioli et al., 2009) (Fig. 7A). Moreover in wild–type plants the majority of the markers were not affected by photoperiodic variations (i.e. they were expressed in a similar pattern either under LDs or SDs) (Fig. 7A). In the *aba1* mutants the expression levels of the majority of these markers was strongly reduced compared with wild type (with the exception of CBF3) (Fig. 7B).

As expected, upon water stress the majority of the tested markers showed a dramatic upregulation independently of the photoperiodic regime (Fig. 7A). Such upregulation was ABA–dependent, as it was strongly reduced in the *aba1-6* background (Fig. 7B).

Next we sought to understand whether the observed ABA–dependent upregulation was somehow controlled by the photoperiod. This was not generally the case, except for *ABI2* and partially *CBF3*, which showed a reduced upregulation upon drought in the SD part of the experiment compared to the LD part (Fig. 7A). To further test this idea we monitored the expression of these ABA marker genes in a *gi* mutant background. The expression of *ABI2* was completely abolished in the absence of functional *GI* while all the other ABA markers displayed somewhat altered expression pattern under control and drought conditions or both (Fig. 7C). Some

markers, including *RD29a* and *CBF3* displayed similar levels of transcript accumulations under normal watering conditions, but no obvious upregulation under drought stress (Fig. 7C). In contrast, *KIN1* and *COR15a* (both downstream targets of CBF3) displayed generally –elevated levels of transcript under normal watering conditions (Fig. 7C). In particular, constitutively high levels of *COR15a* were present in *gi* (in any watering regime), which were similar to that in wild type subjected to water stress (Fig. 7C). Despite the accumulations of higher levels of *KIN1* under control conditions in *gi* compared with wild type, no upregulation occurred under drought conditions (Fig. 7C).

Collectively these data suggest a role for *GI* in the control of these ABA–related genes. Based on this preliminary study, the absence of GI appears to alter the perception of drought stress signalling in Arabidopsis.

FIGURE 1

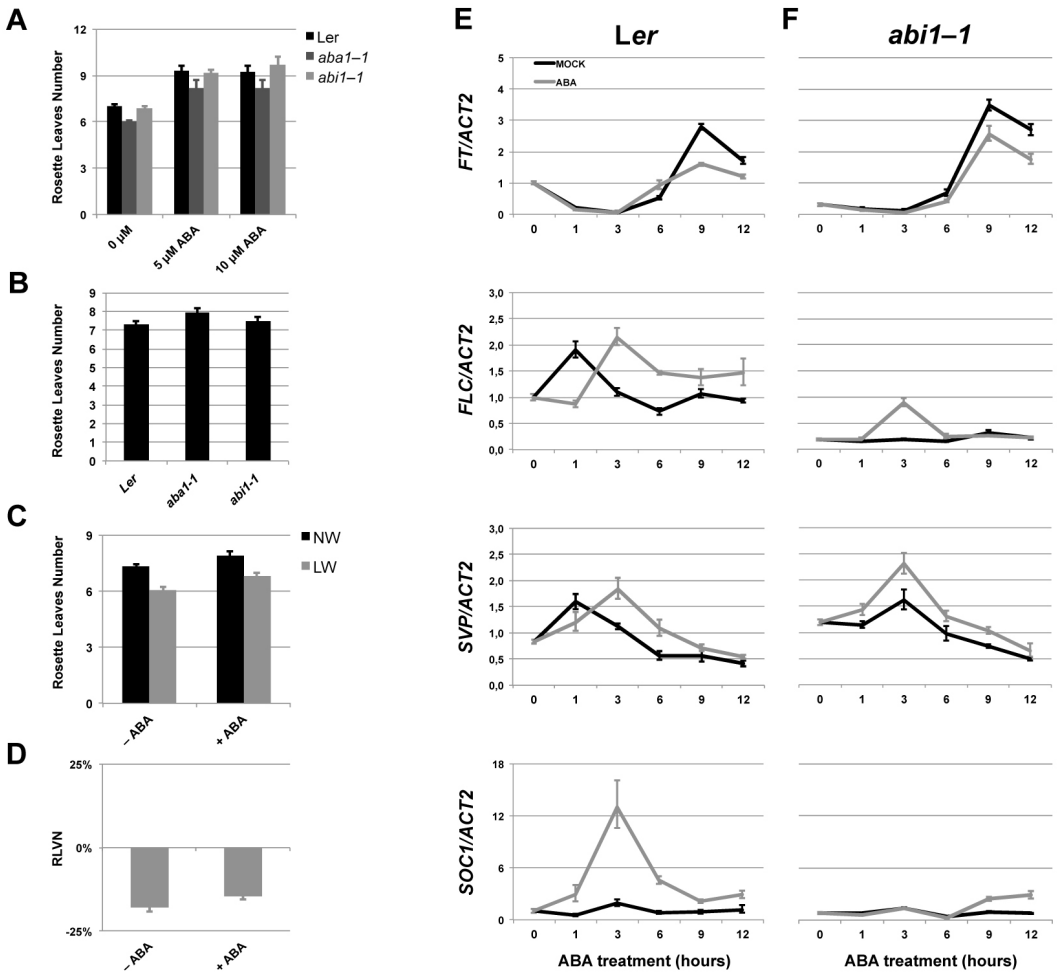

Figure 1. Exogenous ABA delays flowering

(**A**) and (**B**) Rosette leaves mean number of wild type (L*er*), *aba1–1* and *abi1–1* grown on MS supplied with 0, 5 or 10 µM of ABA (**A**) or grown on soil (**B**) under LDs condition. (**C**) Rosette leaves mean number of wild type (L*er*) subjected to normal watering (NW, black bars) or low watering (LW, grey bars) regimes, sprayed with 100µM ABA or mock–sprayed every day. Error bars represent ± SE n = 15. (**D**) Quantification of DE response for mock or

ABA treated wild type plants of (**C**) expressed as relative leaves number variation (RLNV). Numbers indicate percentage variations in number of leaves (%) in plants grown under LW condition relatively to NW. Error bars represent ± SE. (**E**) and (**F**) real–time qPCR of *FT*, *SVP*, *FLC* and *SOC1* transcripts in 7 day–old wild–type (L*er*) (**E**), *abi1–1* (**F**) seedlings upon a shift to 100μM ABA–containing plates. Plants were subjected to mock (black lines) or ABA (grey lines) treatments and harvested at the indicated time points. At each time point, values represent fold change variations of *FT*, *SVP*, *FLC* and *SOC1* transcript levels relatively to L*er* at the start of the treatment. *ACT2* expression was used for normalization; error bars represent SD of two technical replicates.

FIGURE 2

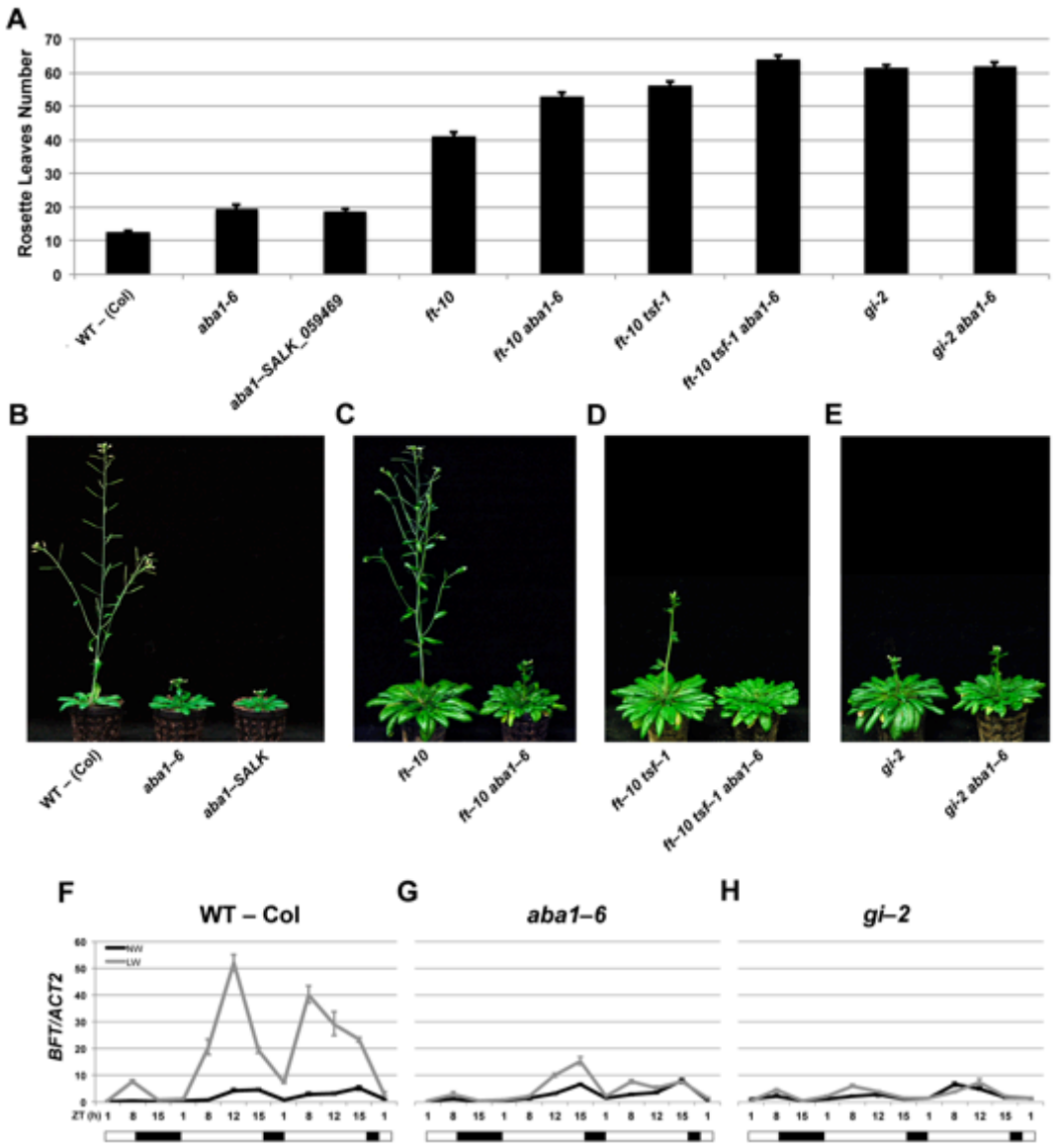

Figure 2. **GI is required for the floral promoting effect of ABA under LDs**

(**A**) Rosette leaves mean number of wild–type (Col–0) and the indicated single and multiple mutants grown under LDs. Error bars represent ± SE n = 15 – 17. (**B**) to (**E**) Images of representative plants of the indicated genotypes detailed in (**A**). Wild–type Col–

0, *aba1–6* and *aba1–SALK* plants are 7 week–old (**B**), *ft–10* and *ft–10 aba1–6* (**C**), *ft–10 tsf–1* and *ft–10 tsf–1 aba1–6* (**D**), *gi–2* and *gi–2 aba1–6* (**E**) are 10 week–old. Scale bars = 1 cm (**F**) to (**H**) real–time qPCR of *BFT* transcripts in 3 week old wild–type (Col–0) (**F**), *aba1–6* (**G**) and *gi–2* (**H**) seedlings. Plants were subjected to NW (black lines) or LW (grey lines) regimes and harvested at the indicated time points in coincidence with the light phase (open bar) or in the dark (black bar) during a SDs to LDs shift. At each time point, values represent fold change variations of BFT transcript levels relatively to Col–0 under NW. *ACT2* expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.

FIGURE 3

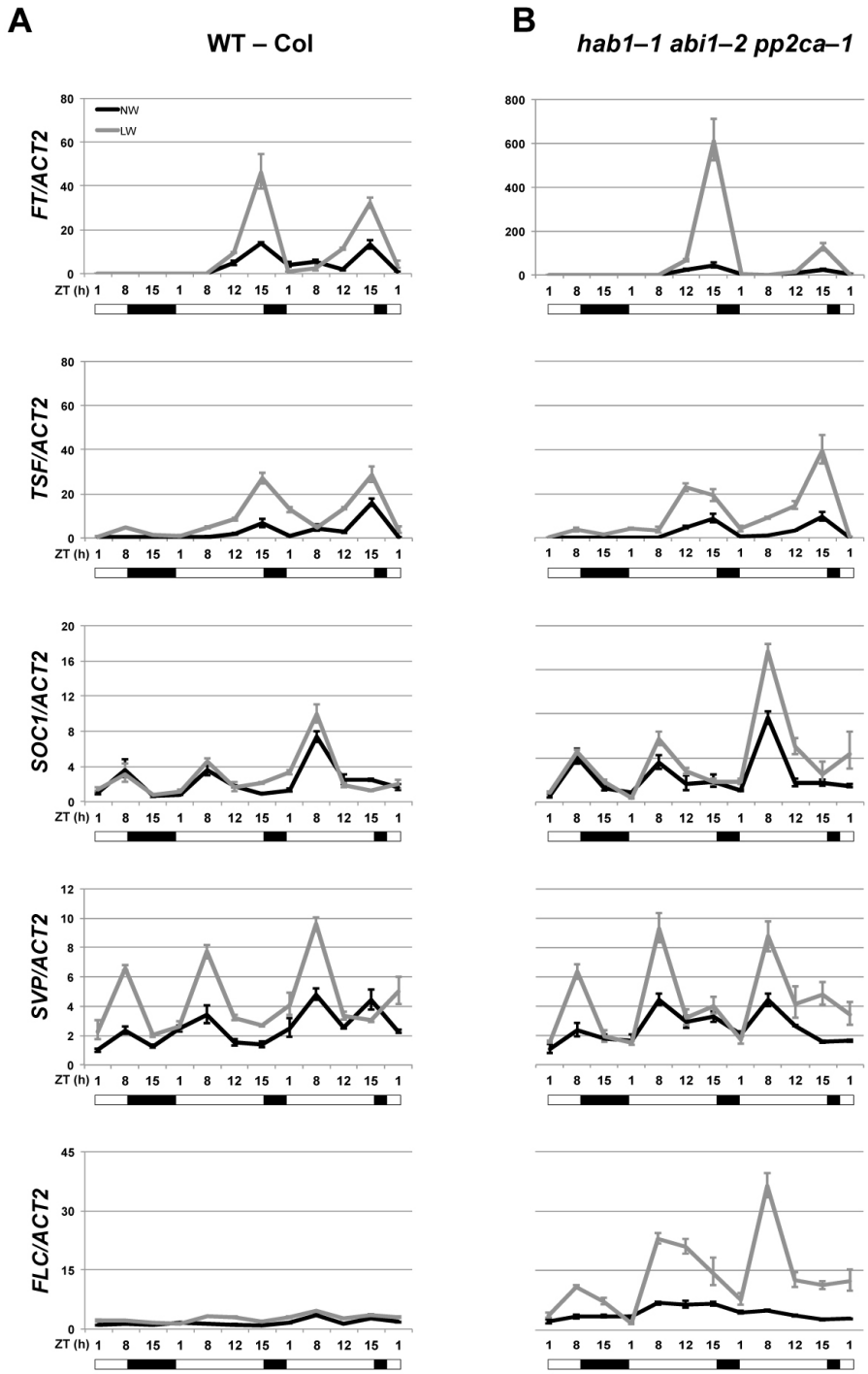
A
WT – Col
B
hab1–1 abi1–2 pp2ca–1
NW
LW
FT/ACT2
TSF/ACT2
SOC1/ACT2
SVP/ACT2
FLC/ACT2
ZT (h) 1 8 15 1 8 12 15 1 8 12 15 1

Figure 3. Enhanced drought– dependent upregulation of *FT* and *FLC* in the *hab1–1 abi1–2 pp2ca–1* mutants.

(**A**) and (**B**) real–time qPCR of *FT*, *TSF*, *SOC1*, *SVP* and *FLC* transcripts in 3 week–old wild–type (Col–0) (**A**) and *hab1–1 abi1–2 pp2ca–1* (**B**) seedlings. Plants were subjected to NW (black lines) or LW (grey lines) regimes and harvested at the indicated time points in coincidence with the light phase (open bar) or in the dark (black bar) during a SDs to LDs shift. At each time point, values represent fold change variations of *FT*, *TSF*, *SOC1*, *SVP* and *FLC* transcript levels relatively to Col–0 under NW. *ACT2* expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.

A

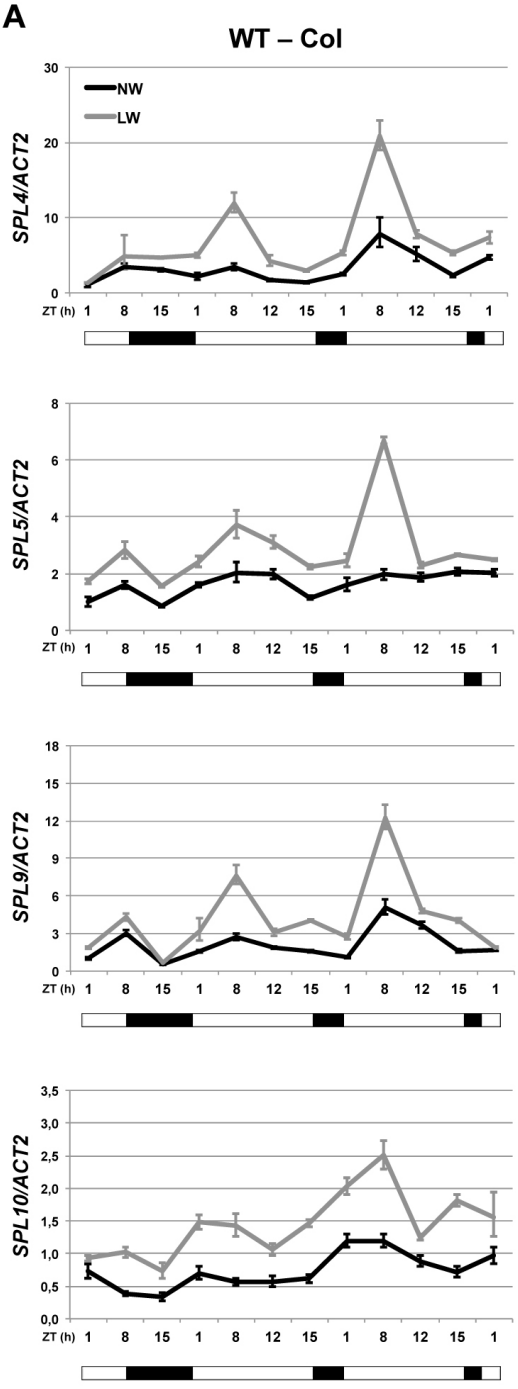

Figure 4. The *SPL* genes are not involved in the drought–dependent floral delay under SDs.

Real–time qPCR of *SPL4*, *SPL5*, *SPL9*, and *SPL10* transcripts in 3 week–old wild–type (Col–0) seedlings. Plants were subjected to NW (black lines) or LW (grey lines) regimes and harvested at the indicated time points in coincidence with the light phase (open bar) or in the dark (black bar) during a SDs to LDs shift. At each time point, values represent fold change variations of *SPL4*, *SPL5*, *SPL9*, and *SPL10* transcript levels relatively to Col–0 under NW. *ACT2* expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.

FIGURE 5

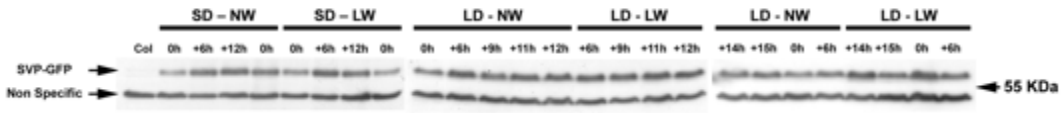

Figure 5. **The SVP protein is not differentially accumulated under drought condition**

Western blot of SVP:GFP fusion protein using an anti–GFP antibody. 3 week old transgenic *SVP$_{pro}$SVP:GFP* plants were subjected to different watering regimes and harvested at the indicated time points during a SDs to LDs shift. Number on the right indicate the molecular mass marker. Non transgenic wild type (Col-0) was used as a negative control. A non–specific, GFP antibodies cross–reacting band (Non Specific) afforded a loading control. Each lane was loaded with 50 micrograms of total proteins.

FIGURE 6

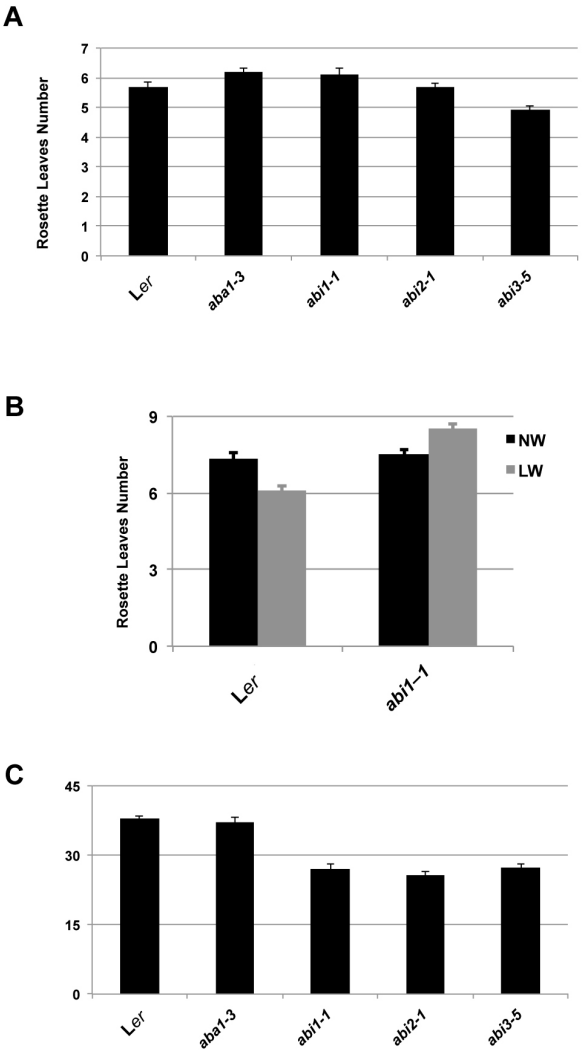

Figure 6. **Different involvement of ABA signalling components in the floral transition**.

(**A**) Rosette leaves mean number of wild type L*er* and relative flowering time mutants grown under LDs. (**B**) Rosette leaves mean number of wild type L*er* and *abi1–1* grown under LDs. Plants were subjected to normal watering (NW, black bars) or low watering (LW, grey bars) regimes. (**C**) Rosette leaves mean number of wild type L*er* and relative flowering time mutants grown under SDs. Error bars represent ± SE n = 15.

FIGURE 7

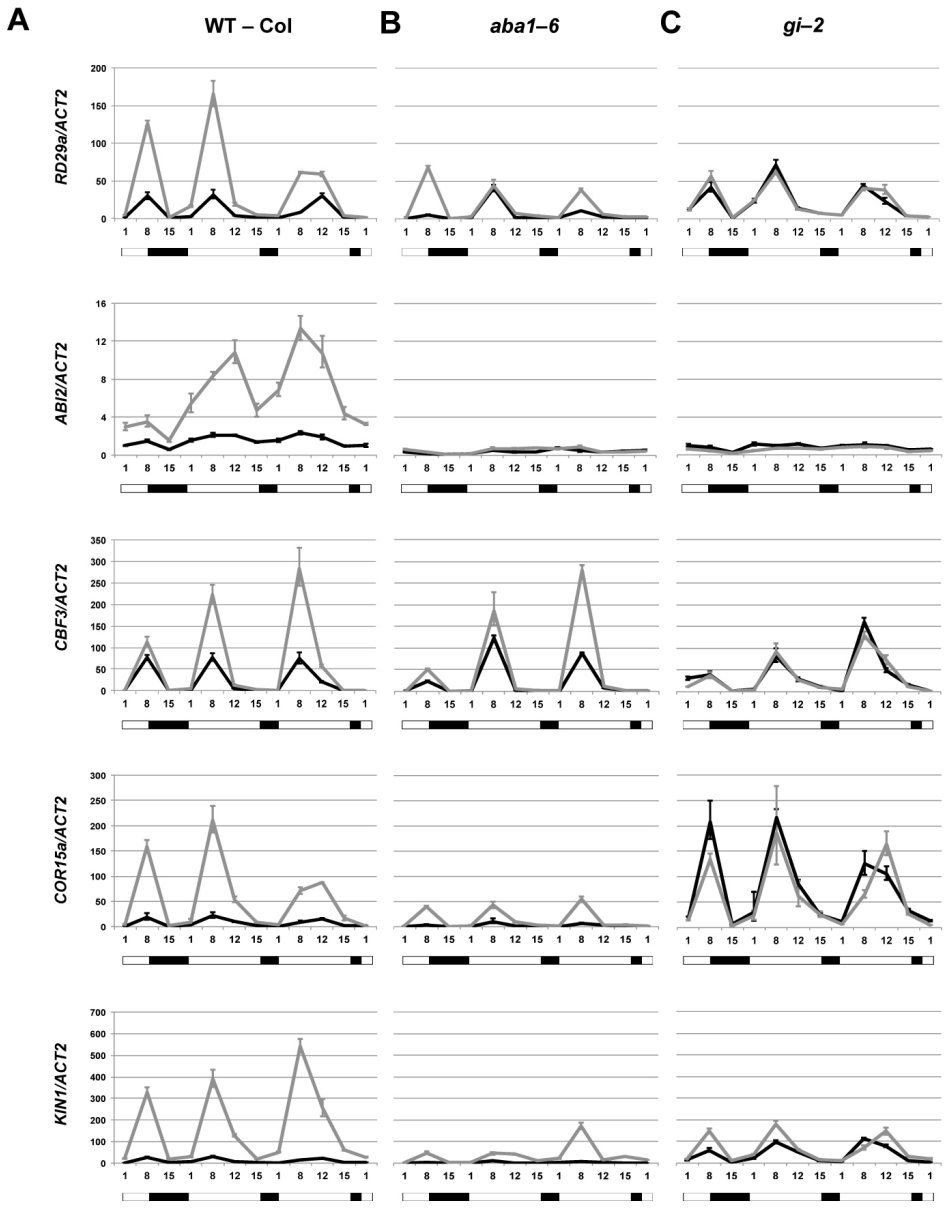

Figure 7. GI regulates the expression of several ABA marker genes.

(**A**) to (**C**) real–time qPCR of *RD29a*, *ABI2*, *CBF3*, *COR15a* and *KIN1* transcripts in 3 week–old wild–type (Col–0) (**A**) and *aba1–6* (**B**) and *gi–2* (**C**) seedlings. Plants were

subjected to NW (black lines) or LW (grey lines) regimes and harvested at the indicated time points in coincidence with the light phase (open bar) or in the dark (black bar) during a SDs to LDs shift. At each time point, values represent fold change variations of *RD29a*, *ABI2*, *CBF3*, *COR15a* and *KIN1* transcript levels relatively to Col–0 under NW. *ACT2* expression was used for normalization; error bars represent SD of two technical replicates. A representative experiment of two biological replicates is shown.

FIGURE 8

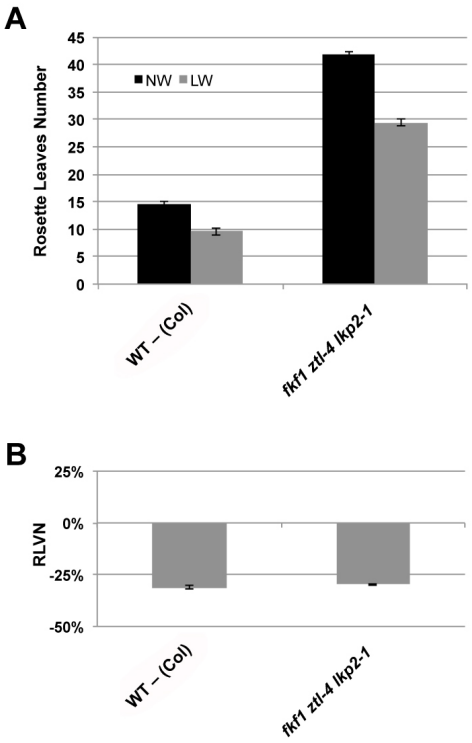

Figure 8. The *ADAGIO* gene family do not participate in the DE response .

(A) Rosette leaves mean number of wild type and the *fkf1 ztl–4 lkp2–1* mutants grown under LDs (16 h light / 8 h dark). Plants were subjected to NW (black bars) or LW (grey bars) regimes. Error bars represent ± SE n = 17.

(B) Quantification of DE response for each genotype detailed in (**A**) expressed as RLNV. Error bars represent ± SE

MATERIALS AND METHODS

In this study we used wild–type *Arabidopsis* plants, ecotype Columbia (Col–0) or Landsberg *erecta* (L*er*). *aba1-3*, *abi2–1* and *abi3–5* mutants (Koornneef, et al. 1982, 1984) were obtained from NASC, *fkf1 ztl-4 lkp2-1* (Fornara et al., 2009) (Fornara et al. 2009) were kindly provided by Dr. Fornara. General growth conditions, SDs to LDs shift experiments, flowering time measurement and quantification of DE response are described in the material and methods of the paper (Part 2)

in vitro plant growth

Seeds were sterilized by 70% ethanol with 0.1% SDS and 0,01 Silwet FASTEX®, rinsed with absolute ethanol and air dried on a filter paper under a flow hood. Seeds were sown on plates containing 0.85% plant agar (Duchefa), 1% Suc and 1x Murashige and Skoog (MS; Duchefa) and incubated for 2d at 4°C to break seed dormancy. Afterward, plants were germinated in LDs growth chamber at 22°C under cool white fluorescent tubes (Osram, Sylvania) at a fluency of 150 $\mu mol\ m^{-2}\ s^{-1}$ (Photosynthetically active radiation).

Exogenous ABA applications

Flowering assay: after germination on MS plates , 3-day-old seedlings were transferred into phytatrays containing MS medium with 0 (mock), or 5 and 10 µM ABA and grown in a growth chamber under LDs conditions until bolting.

Shift assay: after germination on MS plates, 7-day-old seedlings were transferred onto plates containing MS medium with or without 100 µM ABA and grown under LDs. For each time point / treatment / genotype combination, plants were harvested in two biological replicates, each one consisting of approximately 50 seedlings pooled from two different plates and immediately frozen in liquid nitrogen.

Spray assay: 3-d-old seedlings were daily sprayed (approximately 4 h after dawn) with 100 µM ABA or mock until bolting.

Isolation of Double Mutants and Genotyping

Double or triple mutants combinations were generated by crossing. The genotyping of *aba1–6* is described in the material and methods of the paper (Part 2). *gi–2 aba1–6* double mutants were selected for the late flowering phenotype and the presence of the *aba1-6* allele. *ft–10* and *ft–10 tsf–1aba1–6* allele were first selected for the late flowering phenotype and than genotyped. The *ft–10* allele was selected using the SULFADIAZINE resistance carried by the T-DNA and the *tsf1–1* allele was identified by PCR with the following primers: forward 5'-AAGAGAGCAGCAACTTGTCAAG-3', reverse 5'-CGTAGCACACCACCTCATTG-3' for WT allele and Lba1 5'-TGGTTCACGTAGTGGGCCATCG-3' and reverse for the mutant allele.

Protein extraction, SDS-PAGE and Immunoblots

3 week–old transgenic $SVP_{pro}SVP:GFP$ plants were subjected to different watering regimes during a SDs to LDs shift. For each time point, 30 to 40 seedlings were harvested and immediately frozen with liquid nitrogen. Total proteins were extracted using the TRIzol® Reagent (Ambion®) according to the manufacturer's instructions.

Equal amounts (50 µg) of protein extracts were size fractionated on a SDS-PAGE gel and blotted onto a PVDF (Polyvinylidene fluoride) filter for immunoblot. Filters were incubated in TTBS milk (5% [w/v] dry nonfat milk, 25 mM Tris- HCl, pH 8, 150 mM NaCl, and 0.05% [v/v] Tween 20) before incubation with anti-GFP antibodies (Abcam) diluted 1:2000 in TTBS milk. Filters were washed twice in TTBS and incubated with a secondary antibody (anti rabbit, peroxidase–conjugated, Sigma) diluted 1:20,000 in TTBS milk. Filters were washed twice in TTBS and incubated in the peroxidase substrate solution (Millipore) before exposure to film (Hyperfilm ECL; Amersham Pharmacia Biotech).

RNA Extraction and Real–time qPCR

Are described in the material and methods of the paper (Part 2), the primers used are listed below

Gene	Forward	Reverse	Use
SPL4	GTAGCATCAATCGTGGTGGC	CTTCGCTCATTGTGTCCAGC	**qPCR**
SPL5	ATGCAGCAGGTTTCATGAGC	GCCTGACCCTTCTCCAAAAC	**qPCR**
SPL9	TCCTCTTTCAGTGGAGGGCT	TTTGAACGACCACCTGAGGA	**qPCR**
SPL10	TGTTGTGGAATGGGTTGTCC	CCACCAGATGTTTGAAACGC	**qPCR**
KIN1	GCTGGCAAAGCTGAGGAGAA	TTCCCGCCTGTTGTGCTC	**qPCR**
RD29b	ATGGAGTCACAGTTGACACGTCC	GAGATAGTCATCTTCACCACCAGG	**qPCR**
COR15a	CTTACCTAATCAGTTAATTTCAAGCA	TTAAACATGAAGAGAGAGGATATGG	**qPCR**
ABI2	GGAGTGACTTCGATTTGTGGTAGACG	GTCAAAGCCAGATGCATCCTCTCACG	**qPCR**
RD29a	CTTGATGGTCAACGGAAGGT	CAATCTCCGGTACTCCTCCA	**qPCR**
CBF3	TTCCGTCCGTACAGTGGAAT	AACTCCATAACGATACGTCGTC	**qPCR**
BFT	CGCCGGAAACTAGAGAGTGT	GTTGGGCGTTGAAGTAAACA	**qPCR**

Reference

To add:

Reference

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