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The role of ABA in the floral transition: site and mechanism of action

Alice Robustelli Test

PhD Thesis

Scientific tutor: **Dr. Lucio Conti**

Prof. Chiara Tonelli

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Thesis performed at Department of Biosciences
University of Milan
20133 – Milan
Italy

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Abstract (ENG)

The reproductive success of plants depends on their developmental plasticity that is the ability to modulate their growth in response to exogenous and endogenous stimuli. Plants efficiently integrate these signals to coordinate their life cycle according to the best conditions to increase their fitness. Light and water availability is a limiting factor for plants sustenance and growth. In *Arabidopsis thaliana* day length (photoperiod) and water status influence flowering time. In particular, water deficit accelerates flowering thus enabling the drought escape (DE) responses. Interestingly, such DE responses only occurs under inductive long day conditions (LDs, typical of spring and summer seasons) but not short day conditions (SDs) highlighting a link between photoperiod perception and drought responses. The phytohormone abscisic acid (ABA) mediates the DE response, by promoting the upregulation of the florigen genes *FLOWERING LOCUS T* (*FT*) and its paralogue *TWIN SISTER OF FT* (*TSF*), whose expression is activated mainly by LDs. The role of ABA in flowering regulation is controversial as the literature describes both positive and negative roles for ABA in flowering. My PhD work supports the idea that ABA acts as florigen-stimulating molecule under LDs and its activity depends on prior activation of the photoperiodic pathway. I demonstrated that the ABA-dependent activation of *FT* requires GIGANTEA (GI) and CONSTANS (CO) functions, two main components of the photoperiodic pathway that control florigen expression. The generation of transgenic plants over-expressing tagged versions of GI or CO proteins in different ABA genetic backgrounds allowed me to directly asses their activity and measure their accumulations under varying levels of ABA signalling. My results indicate that ABA promotes GI and CO function without affecting their protein stability. An intriguing perspective of my work is that ABA might regulate GI and/or CO accessibility to the *FT* promoter. Further studies are however necessary to test this hypothesis and to decipher the molecular mechanism by

which ABA allow plants to coordinate flowering time according to the prevailing watering conditions.

Abstract (ITA)

Il successo riproduttivo delle piante è da ascrivere alla plasticità del loro sviluppo ovvero la capacità di modulare la propria crescita in risposta a stimoli esogeni ed endogeni. Le piante sono in grado di integrare questi segnali coordinando il loro ciclo vitale e sincronizzando la fioritura in corrispondenza delle migliori condizioni al fine di garantire la conservazione della specie. La disponibilità di luce e acqua è un fattore limitante per la sopravvivenza e la crescita delle piante. In *Arabidopsis thaliana* la lunghezza del giorno (fotoperiodo) e la quantità d'acqua a disposizione della pianta influenzano il tempo di fioritura, nello specifico giorni lunghi (16 ore di luce e 8 ore di buio, condizione tipica della stagione primaverile ed estiva) e siccità accelerano la fioritura. È interessante notare che la fioritura anticipata causata dallo stress idrico (denominata drought escape, DE, letteralmente "fuga dalla siccità") avviene solo quando le piante sono sottoposte a fotoperiodo lungo ma non a fotoperiodo corto (8 ore di luce e 16 ore di buoi). Questo evidenzia che nelle piante esiste un'interazione tra la percezione del fotoperiodo e la risposta alla siccità. Il fitormone acido abscissico (ABA) media l'accelerazione della fioritura in risposta alla siccità promuovendo l'up-regolazione dei geni florigenici *FLOWERING LOCUS T* (*FT*) e il suo paralogo *TWIN SISTER OF FT* (*TSF*), la cui espressione è indotta nei giorni lunghi. Il ruolo dell'ABA nella regolazione della fioritura è tuttavia controverso; in letteratura l'ABA è descritto sia come promotore che come repressore della fioritura. Il lavoro di ricerca svolto durante il dottorato supporta la tesi secondo cui l'ABA è una molecola florigenica, la cui attività dipende dall'attivazione del pathway fotoperiodico. Ho dimostrato che l'aumento ABA-dipendente della trascrizione di *FT* richiede la funzione di GIGANTEA (GI) e CONSTANS (CO), due componenti essenziali del pathway fotoperiodico che controllano l'espressione del florigeno. La generazione di

piante transgeniche in grado di over-produrre le proteine GI o CO fuse ad un epitopo mi ha permesso di studiare l'effetto di tale sovra-espressione di misurare tramite western blot il loro accumulo in risposta all'ABA. I miei risultati indicano che l'ABA promuove l'attività di GI e CO senza alterare la stabilità della proteina. Una prospettiva interessante è che L'ABA potrebbe altresì regolare l'accessibilità di GI e CO al promotore di *FT.* Ulteriori studi sono tuttavia necessari per verificare questa ipotesi e per comprendere il meccanismo molecolare con cui l'ABA permette alle piante di coordinare la fioritura in funzione della condizione idrica.

Part I

1 Introduction

The ability to adapt life cycle and development programs in response to environmental changes and endogenous stimuli is at the base of plants survival. In this context, coordination of flowering time with the best seasonal cues is crucial to ensure high fitness. Accordingly, in agriculture, crop varieties have been genetically selected all over the world in order to maximize their yield at specific latitudes and local environmental conditions (Purugganan and Fuller, 2009).

1.1 The floral transition in *Arabidopsis thaliana*

Arabidopsis thaliana, a flowering plant belonging to the *Brassicaceae* family, is the model species in which the mechanisms underlying the floral transition (the transition from the vegetative phase to the reproductive phase) have been characterized more extensively. In *Arabidopsis*, the floral transition consists of a molecular reprogramming of the shoot apical meristem (SAM). During the vegetative phase, the vegetative SAM produces leaves that are organized in a rosette attached to the soil. After the floral transition, the SAM turns into an inflorescence meristem (IM), it stops producing rosette leaves, the stem elongates (bolts) and the IM generates floral meristems (FMs), which will originate flowers, fruits and seeds (Huala and Sussex, 1993; Sussex, 1989). This transition is irreversible; consequently, the number of rosette leaves produced by the plant before the appearance of floral meristems can be used to evaluate in developmental terms when the floral transition took place in a particular plant or genotype.

1.2 The regulation of the floral transition

Both exogenous and endogenous signals provide plants with information to determine when the floral transition has to take place. The number of hours of light during the day (photoperiod) and experience of cold (vernalization) are the two major environmental cues that trigger the floral transition (Amasino, 2010; Andrés and Coupland, 2012). In parallel, the autonomous and the gibberellic acid (GAs) pathways represent key signalling components conveying endogenous cues (Blazquez et al., 1998; Galvão et al., 2015, 2012; Mutasa-Gottgens et al., 2009; Porri et al., 2012; Simpson, 2004; Wang et al., 2016; Wilson et al., 1992). Additionally, ambient temperature, plant age, biotic and abiotic stresses are other relevant flowering regulators (Huijser and Schmid, 2011; Kazan and Lyons, 2016; Samach and Wigge, 2005; Takeno, 2016). Furthermore, not only GAs but also other phytohormones such as abscisic acid (ABA), jasmonate (JA), brassinosteroids (BRs), ethylene (ET), salicylic acid (SA), cytokinin (CKs) and nitric oxide (NO) participate to regulate flowering in *Arabidopsis* (Achard et al., 2007; Barrero et al., 2005; Conti, 2017; Conti et al., 2014; He et al., 2004; Li et al., 2010; Martínez et al., 2004; Riboni et al., 2016, 2014, 2013; Robson et al., 2010; Y. Wang et al., 2013; Zhai et al., 2015). Interestingly these hormonal signalling pathways seem to converge to a very limited number of floral genes (Davis, 2009; Kazan and Lyons, 2016). Here I will focus my attention on the interaction between the photoperiodic pathway and the role of the phytohormone abscisic acid (ABA) in the regulation of the flowering time (Riboni et al., 2016, 2014, 2013).

1.3 The photoperiodic pathway

Day length perception (photoperiod) has been identified as a crucial environmental stimulus that regulates plant reproductive development since the beginning of the 20th century (Garner and Allard, 1922). Photoperiod is perceived in the leaves where it

promotes the production of mobile signals (the florigens), which are able to move through the vasculature from the leaves to the SAM where they finally trigger the floral transition (Evans, 1971). Depending on the photoperiodic condition that stimulates the floral transition, plants can be divided in three categories: long-day plants, which preferentially flower in spring-summer at temperate latitudes, when the hours of light exceeds a certain threshold (> 14 h); short-day plants that flower under long nights, typical of the tropical and sub-tropical area; day-neutral plants, whose flowering is independent of photoperiod. Arabidopsis is a facultative long-day plant, meaning that it flowers earlier under long day (LDs) compared to short day conditions (SDs) (Mozley and Thomas, 1995). Comprehensive genetic screens in the last decades allowed the identification of mutants in the photoperiodic response. Photoperiod-insensitive mutants are late flowering compared to the wild type under LDs but show no or little flowering time defects under SDs (Fowler et al., 1999; Koornneef et al., 1998, 1991; Putterill et al., 1995). Epistasis analysis demonstrate that photoperiodic flowering mutants can be assigned to the same signalling pathway, which is active under LDs and not under SDs (Kobayashi et al., 1999; Koornneef et al., 1998; Mizoguchi et al., 2005; Suarez-Lopez et al., 2001). *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) constitute the core genes of the photoperiodic pathway. *GI* and *CO* are responsible for photoperiod perception and LDs-dependent activation of the florigenic stimulus whereas *FT*, together with its paralogue *TWIN SISTER OF FT* (*TSF*), represents the mobile florigen that triggers the floral transition at the shoot apex (Corbesier et al., 2007; Golembeski and Imaizumi, 2015; Kobayashi and Weigel, 2007). The photoperiodic pathway is active only under LDs because light-stabilized GI, in complex with FLAVIN-BINDING, KELCH REPEAT, F BOX 1 (FKF1), enables the transcriptional activation of *CO* (Imaizumi et al., 2005; Sawa et al., 2007). Crucially, a peak of *CO* transcripts occurs at dusk, in correspondence with the light phase under LDs conditions. In this temporal window, a series of light-stimulated receptor

promote the stabilization of CO protein, which is otherwise fated for proteasome-mediated degradation (Jang et al., 2008; Song et al., 2012b; Valverde et al., 2004; Zuo et al., 2011). Conversely, under SDs, *CO* transcript peaks in the dark when CO protein stabilization cannot occur (Sawa et al., 2007). A huge number of molecular events regulate photoperiodic signalling via transcriptional and post-transcriptional mechanisms (Andrés and Coupland, 2012; Golembeski and Imaizumi, 2015; Priyanka Mishra, 2015; Song et al., 2014b). Because of this complexity, I will now focus on some key components which are most related to the effects exerted by ABA and drought signals in Arabidopsis.

1.4 *GI* is regulated transcriptionally and post-transcriptionally

GI is a key component of the Arabidopsis photoperiodic pathway and is emerging as an important regulator of several plant environmental responses (Fornara et al., 2015). Despite its importance, it is surprising that very little is known about its molecular function.

Both *GI* transcript and GI protein follow a similar pattern of diel accumulation depending on the photoperiod conditions. Under LDs, *GI* transcript and protein levels are low in the morning and peak 10-12 hours after dawn (zeitgeber time 10 -12, ZT10 -12) while under SDs, the peak is observed at ZT8 (David et al., 2006; Fowler et al., 1999).

It has been demonstrated that components of the circadian clock regulate the transcription of *GI*. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are responsible for *GI* transcript accumulation in a specific time window under LDs thereby *cca1 lhy* double mutants show anticipated *GI* expression (Mizoguchi et al., 2002). As CCA1, other clock proteins, such as LIGHT-REGULATED WD 1 and 2 (LWD1, LWD2) and TIME OF COFFEE (TIC), regulate the rhythmicity of *GI* expression (Hall et al., 2003; Wu et al., 2008). Similar to *cca1 lhy* mutants, *lwd1 lwd2* and *tic* mutants show a peak of *GI* transcript advanced from ZT10 to ZT6. In addition to the

circadian clock, light quality also regulates *GI* expression. For example, under LDs, far-red light delays *GI* transcript accumulation (Wollenberg et al., 2008). Moreover, when *Arabidopsis* plants enter the dark phase, *GI* transcript abundance halves in one hour regardless of their prior photoperiod condition of growth (Fowler et al., 1999). This night time-dependent repression is mediated by the complex EARLY FLOWERING 3 and 4 (ELF3, ELF4) and LUX ARRHYTHMO (LUX) and seems to be associated to a drop in evening temperature (Mizuno et al., 2014).

Although less studied, GI protein also follows a specific pattern of accumulation with a peak at ZT12 under LDs and at ZT8 under SDs (David et al., 2006). In the dark, GI is ubiquitinated by the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) and ELF3 acts as protein adaptor to tether COP1 onto GI (Yu et al., 2008). Conversely, heat shock triggers GI SUMOylation, which prevents GI degradation and consequently promotes flowering acceleration (López-Torrejón et al., 2013).

In the context of flowering time, GI links circadian clock outputs with the photoperiodic pathway by promoting in a specific temporal window the proteasome-mediated degradation of a set of transcriptional repressors that normally occupy the promoter of *CO* and prevent its transcriptional activation (Fornara et al., 2009; Sawa et al., 2007). In line with this model, *gi* loss of function mutants show much reduced *CO* transcript accumulation and undetectable *FT* expression compared to the wild type. However, overexpression of *CO* largely confers early flowering even if *GI* is not functional (Suarez-Lopez et al., 2001). While these genetic studies helped elucidate the important role of GI upstream of *CO*, other studies show that GI can activate flowering independent of *CO*. For example, GI stimulates the expression of *microRNA 172* which targets the *APETALA2* factor *TARGET OF EAT 1*, a repressor of *FT*. Moreover, ectopic expression of *GI* can partially recover the late flowering of *CO* mutants, through restoring in part the transcriptional activation of *FT* (Jung et al., 2007; Sawa and Kay, 2011). Sawa et al.

demonstrated through chromatin immunoprecipitation experiments that GI binds to *FT* promoter regions in proximity to those bound by three *FT* repressors: SHORT VEGETATIVE PHASE, TEMPRANILLO (TEM)1, and TEM2 (Sawa and Kay, 2011). Interestingly, GI physically interact with these factors suggesting a role for GI in altering the competence of the *FT* promoter to receive positive transcriptional regulation through an unknown mechanism.

1.5 The transcriptional and post-transcriptional regulation of *CO*

In Arabidopsis CO constitutes the main activator of the florigen genes expression in LDs photoperiod (Suarez-Lopez et al., 2001). CO induces *FT* transcription by binding directly the *FT* promoter and acting as a transcriptional regulator (Cao et al., 2014; Tiwari et al., 2010).

Several layers of transcriptional and post-transcriptional regulation determine how CO protein accumulates under LDs, which is crucial for its role in triggering the transcription of the florigens. *CO* transcription is indirectly activated by the GI-FKF1 complex. The GI-FKF1 complex assembles only under LDs when *GI* and *FKF1* expression overlap between ZT10 and ZT12 (Imaizumi et al., 2005, 2003; Sawa et al., 2007). FKF1 is a ubiquitin ligase and a blue light photoreceptor (Imaizumi et al., 2003), which forms a complex with GI in a blue light-dependent manner. The GI-FKF1 complex targets the CYCLING DOF FACTORS (CDFs) for their proteasome-dependent degradation (Fornara et al., 2009; Sawa et al., 2007). Because the CDFs are transcriptional repressors of *CO*, their destruction allows for recruitment of other positive regulators onto the *CO* promoter. The transcription factors FLOWERING BHLH 1, 2, 3, 4 (FBH1, FBH2, FBH3, FBH4) bind to the *CO* promoter and stimulate its upregulation at the end of a long day (Ito et al., 2012). Recently, the CINCINNATA (CIN) clade of class II TEOSINTE BRANCHED

1/CYCLOIDEA/PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR (TCP) proteins were shown to act as *CO* activators, by binding to the *CO* promoter. It is interesting to note that TCP4 interacts with GI and that its ability to induce *CO* expression is GI-dependent. GI may thus enhance the DNA-binding ability of TCP4 (Kubota et al., 2017).

The described complex array of transcriptional regulations confer a robust diel pattern of high levels of *CO* transcript accumulation at dusk. Coincidence of high levels of *CO* transcript in the light phase of a LD is essential for the CO protein stabilization and the consequent activation of flowering under LDs. The ubiquitin-ligase complex CONSTITUTIVE PHYTOMORPHOGENIC 1 (COP1) – SUPPRESSORS OF PHYTOCHROME A (SPAs) is responsible for the proteasomal degradation of CO in the dark (Jang et al., 2008; Laubinger et al., 2006). COP1-SPAs activity is repressed by blue light (BL) through the BL photoreceptor CRYPTOCHROME 2 (CRY2) (Zuo et al., 2011). In addition to CRY2 activity, the other BL-photoreceptor FKF1 favours CO protein accumulation in the afternoon (Song et al., 2012b). In this part of the day, PHYTOCHROME A (PHYA), a red/far-red receptor, also participates to stabilize CO (Valverde et al., 2004). Unlike the end of the day, in the morning HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), an E3 ubiquitin ligase, and PHYTOCHROME B (PHYB), a red light receptor, destabilize CO (Lazaro et al., 2012; Valverde et al., 2004). It has been recently demonstrated that the PSEUDO RESPONSE REGULATORS (PRRs), besides regulating *CO* transcription (Nakamichi et al., 2007), also participate to regulate CO protein accumulation during the day (Hayama et al., 2017). All these post-transcriptional events define a coordinated interplay between the endogenous clock and light-activated signals and converge to the stabilization of CO, thus ensuring that its peak of protein accumulation occurs at the end of the day. In addition to CO protein stability control, CO activity and/or degradability may also depend on its phosphorylation

status which varies during the day, thereby the phosphorylated form of CO is more abundant in the light (Sarid-Krebs et al., 2015).

1.6 The transcriptional regulation of *FT*

The access of CO to the CO Responsive Element (CORE) of the *FT* promoter (Cao et al., 2014; Tiwari et al., 2010) is emerging as another layer of control of photoperiodic flowering. In addition to the CORE motifs, which are proximal to the transcription start site, distal cis-elements are required for the transcriptional activation of *FT* by CO. It has been demonstrated that the trimeric NUCLEAR FACTOR - Y (NF-Y) complex binds to a CCAAT-motif 5 kb upstream of the CORE regions (Cao et al., 2014). The formation of a chromatin loop in the *FT* promoter favours CORE and CCAAT motifs juxtaposition; In this way the NF-Y complex can be placed in the vicinity of CO protein to boost its function (Cao et al., 2014). The CORE element (CCACA) is similar but not identical to the well-established NF-Y recognition motif sequence CCAAT (which is not present in the proximal *FT* promoter region). CO protein has been shown to form a non-canonical NF-Y complex which allows CO to divert the NF-YB and C subunits to the CORE sequence (Gnesutta et al., 2017). Formation of this heterogeneous CO/NF-Y complex is functionally relevant since double mutants of *nf-yb nf-yc* subunits phenocopy mutants of *co*. Furthermore, overexpression of CO cannot complement the late flowering phenotype of *nf-yb nf-yc* mutants (Tiwari et al., 2010).

Many other proteins cooperate with CO to stimulate *FT* expression. Among these factors is ASYMMETRIC LEAVES 1 (AS1), which promotes CO-dependent *FT* activation (Song et al., 2012a) and the PHYTOCHROME INTERACTING FACTORS (PIFs) which convey ambient temperature –dependent information onto the *FT* promoter in cooperation with CO (Fernández et al., 2016; Kumar et al., 2012).

Following the transcriptional activation of *FT* in the leaves, FT protein moves through the vasculature to the shoot apex where it forms a complex with FLOWERING LOCUS D (FD), which coordinates the expression of a complex web of floral integrators responsible for the floral specification of lateral primordia (Abe et al., 2005; Corbesier et al., 2007). Moreover, besides *FT*, *Arabidopsis* has other florigen genes including *TWIN SISTER OF FT* (*TSF*) (an *FT* paralogue) (Jang et al., 2009; Yamaguchi et al., 2005) and *MOTHER OF FT* (*MFT*) (W. Kim et al., 2013), which have redundant *FT* function.

1.7 Drought stress and flowering time

As already anticipated, both biotic and abiotic stress influence the time to flowering (Kazan and Lyons, 2014; Takeno, 2016). Among the different environmental factors that influence plant development, the effects of water scarcity are extremely relevant in today's science for their implications with the ongoing climate change. Plants respond to drought stress with three main strategies (Blum, 2005): drought tolerance, dehydration avoidance and drought escape. The drought tolerance response allows plant cells to enter a dormant or semi-dormant state during extremely arid period. However, under mild drought stress, plants tend to minimize water loss and maximize water uptake with a series of physiological strategies globally referred to as dehydration avoidance. Finally, plants can escape from water deficit conditions by synchronising the onset of reproductive development with the rainy season. In temperate climates, characterised by dry summers (e.g. the Mediterranean basin), this usually entails the selection of early flowering genotypes that can effectively escape the summer drought. In some cases, such drought escape strategy (DE) can be adaptive. This means that upon drought stress some plants accelerate flowering, thus ensuring the production of a progeny before the worsening of the environmental conditions. In this adaptive connotation, a DE response strategy has

been described in different species including *Arabidopsis*, *Rice*, *Mimulus*, *Avena barbata* and *Brassica rapa* (Franks, 2011; Ivey and Carr, 2012; Riboni et al., 2013; Sherrard and Maherali, 2006; Xu et al., 2005).

1.8 The drought escape (DE) response in *Arabidopsis*

Previous work in our lab and others have shown a close interaction between DE response and photoperiodic signalling. In particular, the DE occurs only under LDs and not under SDs suggesting that drought signals are somehow integrated with photoperiod perception (Riboni et al., 2013). Indeed, independent genetic and molecular works highlight a clear interaction between drought stress and photoperiodic signalling genes. In essence, the drought-dependent acceleration of flowering is caused by upregulation of the florigen genes *FT* and *TSF* under drought conditions and LDs, but not SDs (Riboni et al., 2016, 2013). *GI* plays a key role in this response. First, because *gi* mutants cannot generate a DE under LDs. Secondly, because the overexpression of *GI* can restore DE under SDs. Thirdly, without *GI*, water deficit cannot reactivate *FT* and *TSF* expression (Riboni et al., 2013). My published data demonstrate that *CO* is also essential for the drought-dependent boost of *FT* transcript levels, but not *TSF* (Riboni et al., 2016) indicating that GI can have CO-independent functions in DE.

Besides requiring an activated photoperiodic pathway, the DE response depends on the hormone ABA (Riboni et al., 2016, 2013). Mutants deficient in ABA production or signalling cannot activate DE under LDs and this is accompanied with highly diminished upregulation of the florigen genes in response to drought. Not only these observations indicate ABA as a critical component of the DE response, but they also imply a general involvement of ABA signalling in the control of flowering via regulation of the florigen genes, which has not been previously characterised.

1.9 ABA and flowering

ABA is the key drought stress-related hormone (Shinozaki and Yamaguchi-Shinozaki, 2007). However, ABA controls several plant developmental processes also in the absence of stress (Barrero et al., 2005; Liu et al., 2016). The ABA signalling pathway has been recently characterized and consists of three main components: the ABA receptors PYRABACTIN RESISTANCE (PYR)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR); a group of PP2C phosphatases of group A that act as ABA-signalling repressors (PROTEIN PHOSPHATASE 2Cs, PP2Cs); the kinases SNF1-RELATED PROTEIN KINASES 2 (SnRK2s), which act as ABA-signalling promoter of downstream responses (Cutler et al., 2010). In the presence of ABA, ABA-bound PYR/PYL/RCAR receptors interact with PP2Cs proteins (such as ABI1, ABI2, HAB1, PP2CA) repressing their activity, thus releasing the function of downstream SnRK2 kinases. As a consequence, the kinases autophosphorylate and phosphorylate their targets, among which several transcription factors (Furihata et al., 2006; Umezawa et al., 2013; P. Wang et al., 2013; Y. Wang et al., 2013; Yoshida et al., 2014). The diagram in Fig. 1 illustrates key events of ABA biosynthesis and signalling.

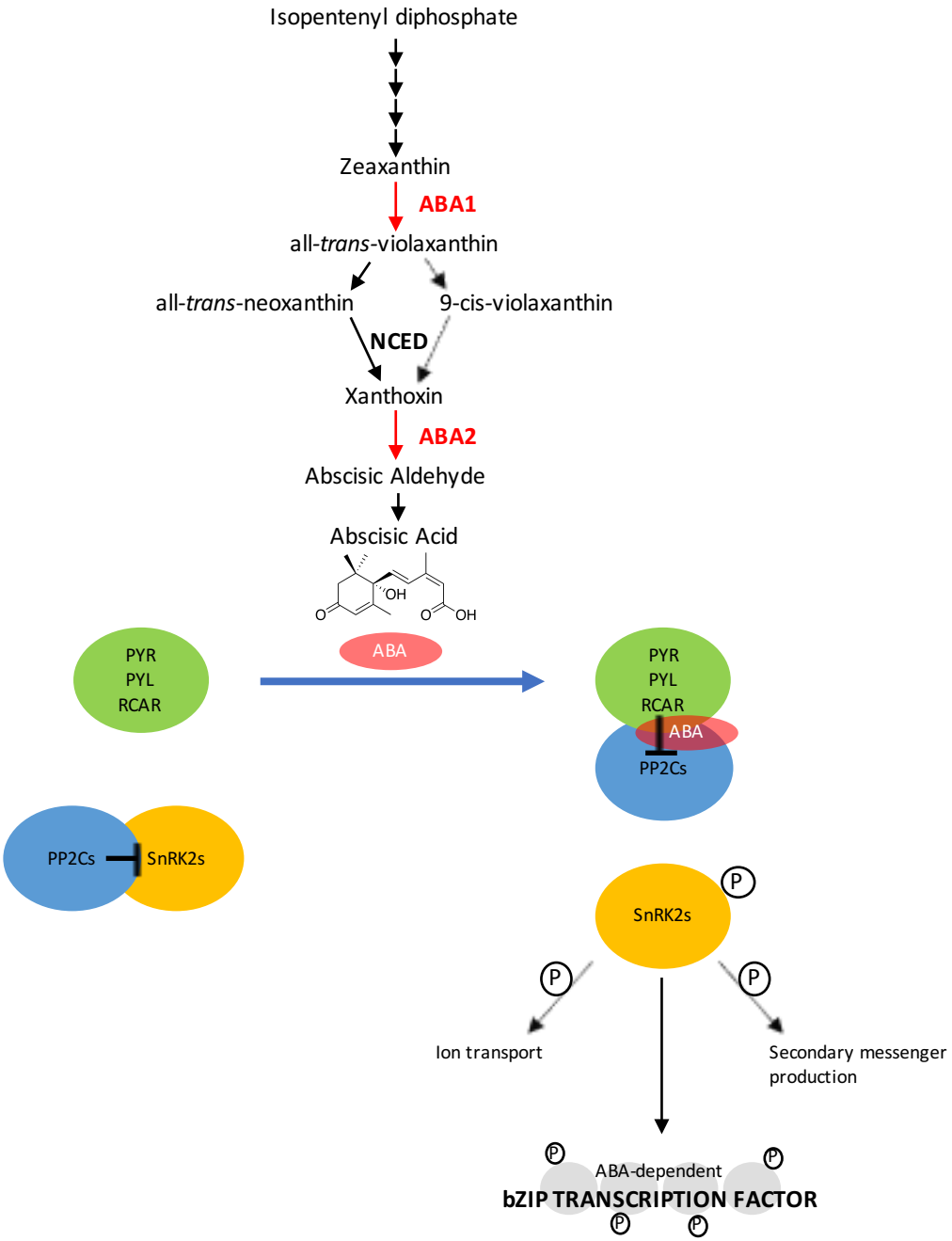

Fig. 1. ABA biosynthesis and ABA signalling. The upper part of the diagram illustrates the ABA biosynthesis. Highlighted in red are two genes that I included in my genetic analysis (*ABA1* and *ABA2*) and the respective reactions, which are compromised in *aba1* and *aba2* mutants. The lower part depicts the phosphorylation cascade of the ABA signalling pathway, which starts when ABA binds to the PYR/PYL/RCAR receptors. Following ABA-induced conformational changes, the receptors sequestrate the PP2Cs phosphatase blocking their activity; consequently, SnRK2s kinase are free to autophosphorylate and phosphorylate their targets.

The role of ABA in the control of the floral transition is twofold as positive and negative effects were reported (Domagalska et al., 2010; Riboni et al., 2016, 2013; Y. Wang et al., 2013). In line with the idea of ABA acting as floral promoter, *Arabidopsis* mutants defective in ABA production are late flowering compared with the wild type under LDs but show no defect under SDs (Riboni et al., 2016, 2013). Root applications of ABA promote flowering under LDs (Koops et al., 2011; Riboni et al., 2016) and rescue the delay in flowering of *aba1* and *aba2* mutants in *Arabidopsis* (Riboni et al., 2016). Such positive effect of ABA on flowering is likely mediated by the canonical ABA signalling pathway. First, because triple *pp2ca* mutants, characterised by hyper activated ABA responses, are early flowering compared to the wild type under LDs (Riboni et al., 2013). Secondly, because higher order mutants of ABA-related *bZIP* transcription factors are late flowering under LDs (Yoshida et al., 2014). Thirdly, because ABA promotes the degradation of *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) transcription factor, which is a flowering repressor (Kurup et al., 2001; Zhang et al., 2009). Besides activating flowering, ABA has been described in the literature as a floral repressor. For example, drought stress delays flowering under SDs (Riboni et al., 2013) and in the same photoperiodic conditions mutants with an enhanced or repressed ABA signalling are late and early flowering, respectively (Riboni et al., 2016, 2013). Such negative effect of ABA in flowering might be exerted through downregulation of *SOC1* expression, independent of *FT* (Riboni et al., 2016). Moreover, *ABSCISIC ACID INSENSITIVE 5* (*ABI5*), an ABA-related bZIP transcription factor, represses flowering through upregulation of the flowering repressor *FLOWERING LOCUS C* (*FLC*) (Y. Wang et al., 2013). It has been proposed that this double effect of ABA on flowering could be related to tissue specificity, whereby ABA has a positive role in the leaf under inductive LDs via boosting florigen expression and a negative role in the shoot apex via *SOC1*

which is always present but clearly emerges under non photo-inductive conditions (Riboni et al., 2016).

If we consider ABA as floral promoter, this activity is highly interconnected with photo stimulated photoperiodic signalling (Riboni et al., 2016, 2013). Indeed, there is a clear genetic interaction between ABA production and the florigen genes since without *GI* or *CO* the drought-dependent upregulation of *FT* cannot occur and both *gi* and *co* mutations are epistatic to ABA deficiency (Riboni et al., 2016, 2013). Also, impairing ABA signalling suppresses the strong activation of *FT* and *TSF* conferred by overexpression of *GI* (Riboni et al., 2016, 2013). ABA production is highly active in the phloem companion cells (where *CO* is usually active) (Kuromori et al., 2014). This might indicate a general interaction between ABA and the photoperiodic genes in the control of different drought-dependent responses. The emerging role of *GI*, *FT* and *TSF* in promoting stomata aperture, which is related to transpiration, supports this general idea (Ando et al., 2013).

2 Motivation and objectives of my PhD: revealing the mode of interaction between ABA and photoperiod signalling

Although data indicate that ABA acts upstream of the florigen genes, it is not clear how it interacts with *CO* and *GI*. My published data demonstrate that ABA mainly promotes GI and CO functions rather than their transcriptional activation (Riboni et al., 2016). Several lines of evidence support this conclusion. First, although my study and data elsewhere support a positive role for ABA in the transcriptional activation of *CO* (Riboni et al., 2016, 2013; Yoshida et al., 2014), in my experience the positive effect of ABA on *CO* transcriptional activation is particularly evident only in mutant plants with enhanced ABA signalling under drought stress (Riboni et al., 2016). Conversely, no clear *CO* upregulation is observed in ABA hypersensitive mutants under normal watering conditions, which instead show highly increased levels of *FT* transcript relative to the wild type (Riboni et al., 2016). Moreover, plants defective in ABA production or signalling have lower levels of florigen transcript compared with the wild type, whereas the expression of *GI* and *CO* is only mildly affected (Riboni et al., 2016, 2013). Secondly, in mutant backgrounds characterised by high levels of *CO*, water deficit can further boost *FT* and *TSF* transcription at dusk without causing a correspondent increment in *CO* (Riboni et al., 2016). Despite the key role of CO in regulating *FT* expression high levels of *CO* are not sufficient to activate the florigen genes under drought stress conditions, as this requires functional GI protein (Riboni et al., 2016). ABA-derived signals thus appear to be largely integrated in the photoperiodic pathway primarily through GI and downstream of *CO* transcriptional regulation. This observation adds to the increasing number of post-transcriptional regulatory mechanisms of GI and CO functions that contribute to the transcriptional regulation of the florigen genes (Cao et al., 2014; David et al., 2006; Hayama et al., 2017; Lazaro et al., 2015; Sarid-Krebs et al., 2015; Song et al., 2014a,

2012a, 2012b; Valverde et al., 2004; Yu et al., 2008; Zuo et al., 2011). Based on these observations, my current hypothesis is that ABA exerts a post-transcriptional regulatory role in photoperiod signalling, thus explaining the ABA-mediated *FT* upregulation that cannot be justified solely by *CO* transcriptional changes.

During my PhD work I contributed to substantiate the idea that ABA regulates GI and CO functions, through a blend of genetic and biochemical approaches (Riboni et al. 2014; Riboni et al. 2016, Unpublished data). Based on published data, in Fig. 2 I illustrate a hypothetical model of interaction between ABA and the photoperiodic pathway whereby ABA mainly regulates GI and CO activities upstream of *FT*. A second aim of my PhD project was to decipher the molecular mechanism by which ABA promotes florigen genes expression through the analysis of CO and GI protein functions and accumulations *in vivo*.

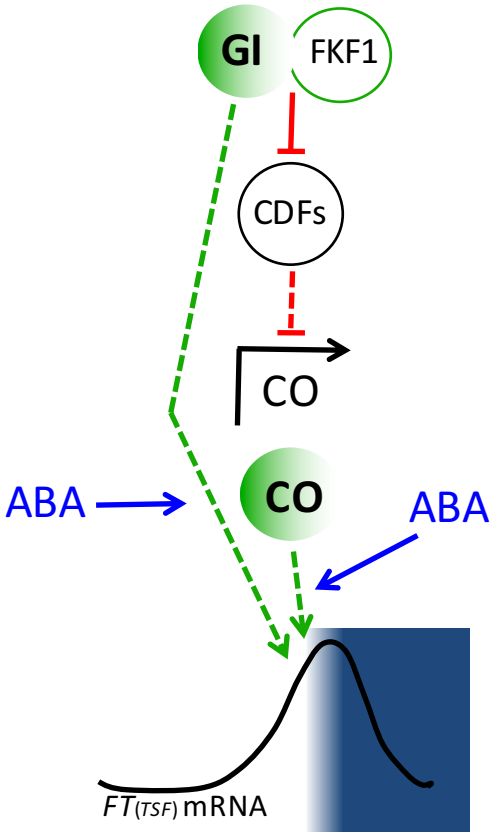

Fig. 2. ABA regulates GI and CO functions rather than their transcriptional activation. Under LDs activation of the photoperiodic pathway depends on the GI/FKF1-mediated degradation of CDFs, which are repressors of *CO*. CO protein is stabilized at dusk, and this promotes florigen genes upregulation. Blue arrows indicate two possible modes of ABA regulation of GI and CO functions. Dashed lines represent transcriptional regulation events, whereas solid lines indicate post-transcriptional controls. Arrows and blunt lines indicate promotive and repressive effects, respectively.

3 Unpublished data

3.1 Main Results

Genetic interactions between ABA production and photoreceptors signalling

A hallmark of photoperiodic signalling is the coincidence between high levels of *CO* transcript and the presence of light in the late afternoon (Suarez-Lopez et al., 2001). This overlap allows for CO protein stabilization and transcriptional activation of *FT* (Jang et al., 2008; Liu et al., 2008; Song et al., 2012b; Valverde et al., 2004; Zuo et al., 2011). In this context, light quality plays a major role in the regulation of CO stability during the day. Red light (RL) destabilises CO in the morning whereas blue (BL) and far-red light (FRL) promote CO accumulation at dusk (Liu et al., 2008; Song et al., 2012b; Valverde et al., 2004; Zuo et al., 2011). To understand which photoreceptor could mediate ABA function upstream of CO I used a genetic approach to combine mutants deficient in ABA production with mutants defective in photoreceptor function. In this set of experiments, I used *phyB* and *phyA* mutants defective in RL and FRL perception, respectively, and *fkf1* and *cry2* mutants deficient in BL sensing. I then analysed the flowering time of the different double mutants in several independent experiments under LDs. As expected, mutants of *aba1-6* were late flowering compared to the wild type (Fig. 3A and 3B). PhyB negatively affects CO accumulation in the morning (Valverde et al., 2004). *phyB-9* were indeed earlier flowering compared to the wild type (Fig. 3A). In two independent experiments *aba1-6 phyB-9* double mutants flowered with a similar number of rosette leaves of *phyB-9* single mutant plants (Fig. 3A and 3C). I excluded that the early flowering conferred by *phyB-9* could mask the effect of *aba1-6* in flowering because in other experiments the *aba1-6* mutation could significantly rescue the early flowering conferred by the *elf3-1* mutation, which is even more severely early flowering compared with *phyB* (Riboni et al., 2016). This

data indicates that *phyB* is epistatic to *aba1-6*, pointing to possible negative role for ABA in the red light-mediated degradation of CO (Lazaro et al., 2015).

To further test the interaction between ABA and red light I analysed mutants of *phyA*, a phytochrome which is more specific for FRL (Neff et al., 2000). PhyA contributes to the CO stabilisation in the late afternoon, when ABA is predicted to act (based on the effect on *FT* accumulation) and *phyA* mutants are late flowering under LDs compared with the wild-type (Johnson et al., 1994; Valverde et al., 2004). I used the *phyA-501* null allele for my experiments (Ruckle et al., 2008) and I consistently observed a significant delay in flowering compared with the wild type (Fig. 3B and 3C). *aba1-6 phyA-501* double mutants were later flowering compared with *phyA-501* (Fig. 3B and 3C). I obtained similar results in repeat experiments, in which *aba1-6 phyA-501* generated approximately 32% (n=3 independent experiments) leaves more compared with *phyA-501.* The additive effect of *aba1-6* and *phyA-501* mutations suggests that FRL and ABA promote flowering through independent mechanisms.

Similar to FRL, BL stabilises CO at dusk (Song et al., 2012b; Valverde et al., 2004; Zuo et al., 2011). Because the effects of varying ABA levels on *FT* transcription were limited to dusk (Riboni et al., 2016, 2013), I focused my analysis on the role of BL-dependent signals. BL is involved in the transcriptional activation of *CO* through the *FKF1* photoreceptor (Imaizumi et al., 2005). BL also promotes CO stabilization through FKF1 and CRY2 functions (Song et al., 2012b; Zuo et al., 2011). *fkf1-10* and *aba1-6 fkf1-10* double mutants were similarly late flowering in all the experiments performed (Fig. 3A and 3C). This data is consistent with ABA acting through *CO*, upstream of the florigen genes. However, I interpret this result with caution since without *FKF1* function the accumulation of *CO* transcript is compromised, which may mask the putative effect of the deficit in ABA production on CO protein function (Imaizumi et al., 2005). In this respect, a more informative BL photoreceptor could be CRY2, which affects CO protein function by

regulating its rate of turnover (Zuo et al., 2011). To test the role of CRY2 in the ABA-dependent activation of CO function, I have generated double mutants of *aba1-6 cry2-1.* This genotype produced highly conflicting results, which precluded a clear definition about the interaction between ABA and *CRY2*. In three independent experiments, I have observed contrasting phenotypes for *aba1-6 cry2-1* as compared with *cry2-1* single mutants. These fluctuations of flowering time were in both directions (later and earlier) and highly significant (10% or 15% increase or decrease in leaf number, respectively). In one case, I observed no variations, which could be interpreted in terms of an epistatic interaction between *CRY2* and ABA production. I also noticed that *aba1-6 cry2-1* mutants were extremely susceptible to diseases (powdery mildew) and pathogen attacks which compromised plant development and growth. Besides generating the *aba1-6 cry2-1* I also produced the *aba1-6 cry2-1 cry1-2* triple mutants but flowering time data have not been obtained yet (also because of the above reasons). However, although CRY1 acts redundantly with CRY2 with respect to a subset of BL–mediated responses, the literature suggests that its role in flowering time regulation is marginal as compared with CRY2 (Bagnall et al., 1996; Guo et al., 1998).

In conclusion, my genetic analysis pointed to a possible interaction between ABA and RL and also between ABA and BL. In contrast, FRL signals seem to act additively to ABA.

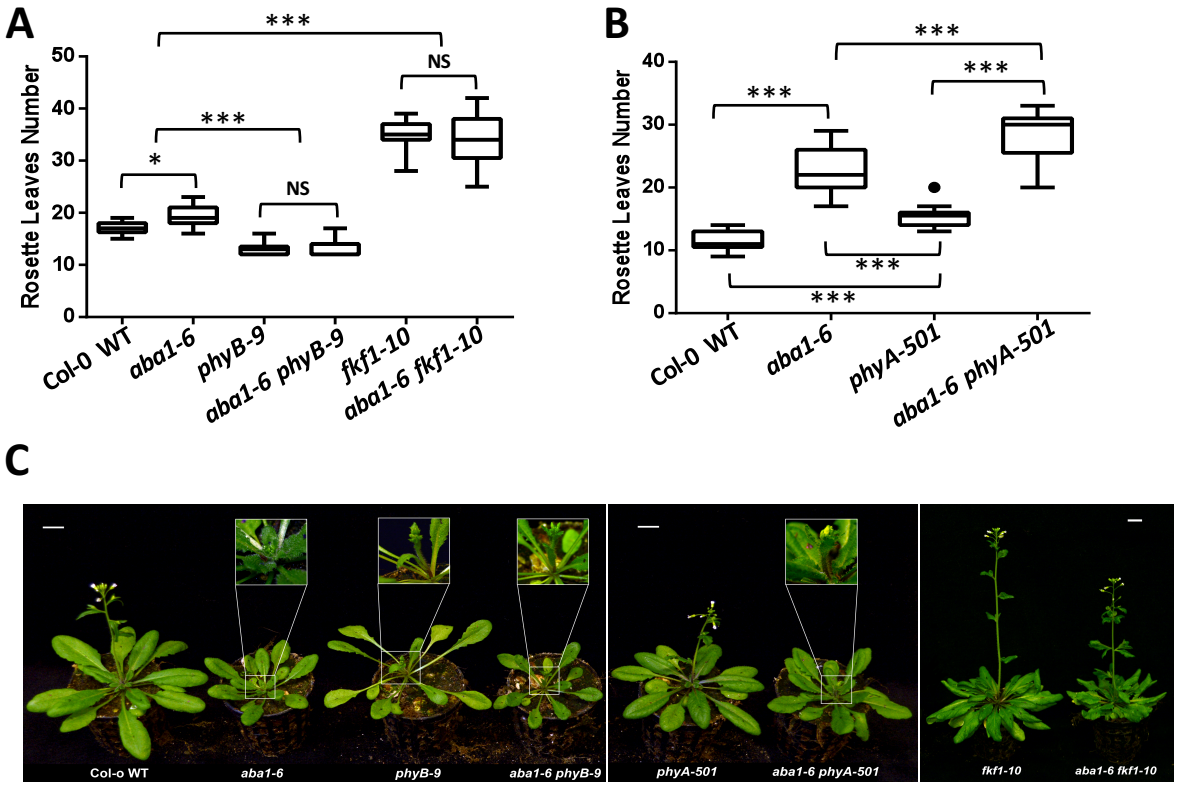

Fig. 3. Blue/Red/Far-Red photoreceptors are differentially involved in the ABA–dependent regulation of flowering. (A-B) Tukey boxplot of the distribution of the rosette leaves numbers of the indicated genotypes grown under LDs (n= 15-17 plants for each genotype). Box extends from the 25th to 75th percentiles and the horizontal line is the median value. Whiskers and dots are calculated following the Tukey method: if the highest value in the data set is lower than (or equal to) the 75th percentile plus 1.5IQR (IQR is the interquartile difference, the difference between 75th and 25th percentiles), the upper whisker represents the maximum value, otherwise the upper whisker stops to the highest value less than 75th percentile plus 1.5IQR and all other values greater than this are drawn as dots (outliers). The opposite is true for the lower whisker: if the lowest value in the data set is higher than (or equal to) the 25th percentile minus 1.5 IQR, the lower whisker represents the minimum value, otherwise the lower whisker stops to the lowest value greater than 25th percentile minus 1.5IQR and all other values lower than this are represented as dots. Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.05 (*), ≤0.001 (***), >0.05 not significant (NS). Experiment (A) and experiment (B) were performed in different growth chambers and in (A) the *aba1-6* mutants had milder late flowering phenotypes compared with the wild type (Col-0). **(C)** Images of representative plants of the indicated genotypes grown under LDs. Col-0, *aba1-6*, *phyB-9* and *aba1-6 phyB-9* are four weeks old, *phyA-501* and *aba1-6 phyA-501* are 5-week-old and *fkf1-10* and *aba1-6 fkf1-10* are seven weeks old. Inset pictures show visible inflorescences. Scale bars= 1cm

Reduced ABA accumulation impairs the function of CO upstream of *FT*

Published data support both transcriptional and post-transcriptional effects for ABA in the activation of *CO* under LDs (Riboni et al., 2016, 2013; Yoshida et al., 2014). However, our data argue in favour of a major post-transcriptional effect of ABA on CO protein.

To prove that ABA promotes CO function and to identify the potential post-transcriptional regulation mechanism exerted by ABA on CO, I decided to generate transgenic plants constitutively expressing *CO* in different mutant backgrounds characterised by different degrees of ABA content and ABA signalling. If the post-transcriptional activity of ABA is crucial as initially hypothesised, a deficit in ABA production would compromise *FT* expression despite overexpression of *CO*. Using the gateway system, I first cloned *CO* under the *Cauliflower Mosaic Virus* (*CaMV*) *35S* promoter, for its ectopic expression in the plant, and with a fluorescent tag at the C-terminus to allow for detection of CO protein (Fig. 4A). With this construct I transformed wild-type plants and *aba1-6* mutants (deficient in ABA production), hereafter referred to as *35S::CO:CFP* and *aba1-6 35S::CO:CFP*, respectively. As a control, I used wild-type plants transformed with the empty gateway (GW) destination vector *35S::GW:CFP* (referring to them as Col-0 *35S::vector*). Basta-resistant T1 plants were selected under standard LD conditions and these were scored for flowering time by counting the number of rosette leaves (26-28 plants for each genotype). This allowed me to accurately evaluate the effect of the overexpression of *CO* on flowering time depending on the ABA background and (given the high number of T1 events) to control for insertional position effects across the different transgenic events. Although I observed high phenotypic variability, *35S::CO:CFP* plants were significantly earlier flowering compared to *Col-0 35S::vector* control plants. In contrast, *aba1-6 35S::CO:CFP* were later flowering either than *Col-0 35S::vector* and *35S::CO:CFP* (Fig. 4B). Therefore, the over expression of *CO* cannot rescue the delay in flowering caused by reduced ABA production. To ensure that the phenotypes observed did not derive from varying levels of transgene-dependent overexpression of *CO*, I measured the transcript levels of *CO:CFP* in around half of the *35S::CO:CFP* plants both in the Col-0 and *aba1-6* backgrounds. Since the flowering time was highly variable within the same genotype, I analysed plants with different rosette leaves number to exclude any bias. I sampled the fourth leaf of bolted

plants at ZT12, when *CO* normally peaks to trigger *FT* expression. Real time qPCR analysis confirmed that the *CO* overexpression (as shown by *CFP* expression) was similar, independent of the ABA content of the plants (Fig. 4C). This pattern of accumulation of *CO:CFP* was confirmed when using *CO*-specific primers in a subset of lines (data not shown). Despite there were no obvious alterations in *CO:CFP* transcript accumulation, *aba1-6 35S::CO:CFP* showed a general strong reduction in *FT* transcript accumulation compared with *35S::CO:CFP* in almost every single transformation event (Fig. 4D). In a subset of individuals, I evaluated the expression values of *CO:CFP* and *FT* with two additional housekeeping reference genes, *ACTIN 2* (*ACT2*) and *IRON-SULFUR CLUSTER ASSEMBLY PROTEIN 1* (*ISU1*) (Kaiserli et al., 2015) and I obtained comparable results to *IPP2*, thus reinforcing my gene expression analysis (data not shown). I did not observe any clear correlation between *CO* and *FT* transcript levels, neither in the wild type nor in *aba1-6* backgrounds. Instead I observed a correlation between the levels of *FT* transcript accumulation and the genotype, thereby active ABA production, as in the Col-0 wild-type background, conferred high *FT* transcript levels. This was apparent when plotting *FT* as a function of *CO:CFP* expression; two clusters of T1 plants appeared, corresponding to the wild-type and the ABA-deficient backgrounds (Fig. 4E). Such highly clustered distribution of gene expression supports the hypothesis that reduced levels of ABA compromise *FT* upregulation even when *CO* is constitutively expressed.

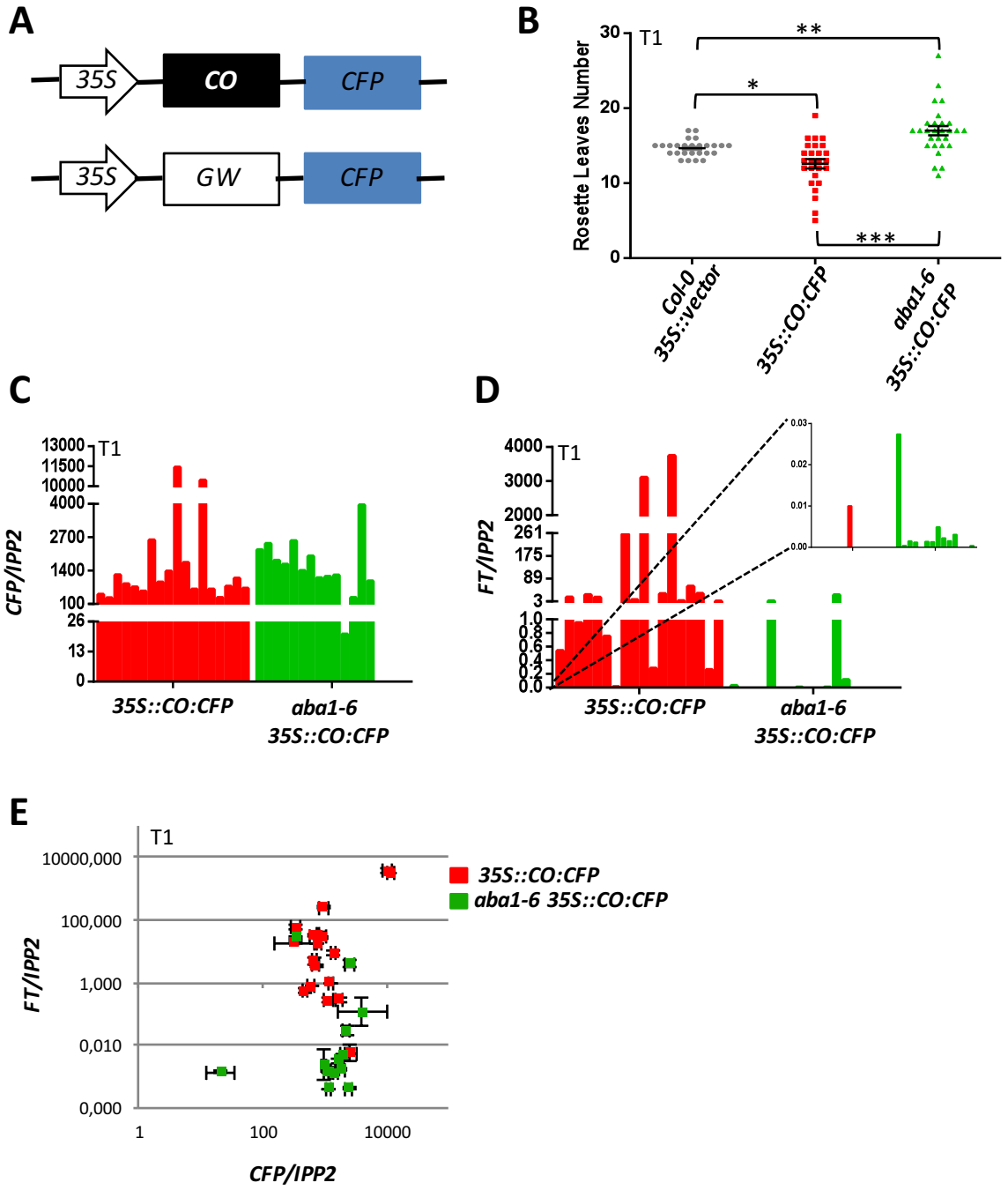

Fig. 4. Reduced ABA impairs *FT* activation even when *CO* is overexpressed. (A) Diagram illustrating the main regulatory elements and tags in the constructs used for *Agrobacterium*-mediated transformation of *Arabidopsis*. **(B)** Scatter plot representing the rosette leaves number of transgenic T1 plants grown under LDs. Each dot represents an independent T1 plants. Error bars are SD, n = 26-28. Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.05 (*), ≤0.01 (**), ≤0.001 (***). **(C-D-E)** Real-Time qPCR of *CO:CFP* and *FT* transcripts in mature (bolted) T1 plants sampled at ZT12. Each column or dot represents gene expression levels of independent T1 plants. *IPP2* expression was used for normalization. **(C and D)** Values represent fold change variations of *CO:CFP* and *FT* transcripts normalized relative to a *35S::CO:CFP* line with low *CO:CFP* expression. For each sample, two technical replicates were performed and the associated standard deviation was omitted for graphic clarity. Inset shows *FT* values lower than 0.03. **(E)** The same values shown in (C-D) are represented in a scatter (XY) plot where *FT* transcript levels are compared with *CO:CFP*. Each dot corresponds to independent T1 plants and error bars represent the standard deviation of two technical replicates.

I next aimed to confirm these results in the subsequent generations. To obtain stable T3 lines I preferentially selected T1 plants with a single transgene insertion, on the basis of the appropriate 3:1 (resistant vs. sensitive) Mendellian segregation of Basta resistance in the T2 generation. However, I also decided to move forward lines with multiple insertions which showed an early flowering phenotype. This was done because of the paucity of very early flowering individuals in T1. Secondly, it is very well established in the literature that the over-expression of CO causes early flowering, which excluded any bias in my choice (An et al., 2004; Jang et al., 2009; Onouchi et al., 2000). I then analysed the flowering time of these segregating (or not) T2 lines (17 plants for each line) to confirm the inheritance of the phenotypes conferred by the transgene. Independent *aba1-6 35S::CO:CFP* lines were still consistently later flowering compared with *35S::CO:CFP* although a great variability in flowering within each line and across genetic backgrounds was observed. In most cases, T2 *35S::CO:CFP* plants were earlier flowering compared with *aba1-6 35S::CO:CFP* but no dramatic early flowering phenotype was observed (Fig. 5A). After confirmation that the *CO:CFP* transgene was still detectable, I also confirmed overexpression of *CO* with gene-specific primers (relative to a non-transgenic wild-type at a similar stage) (Fig. 5B and 5C). In the T3 generation, I decided to focus my attention on two particular lines, *35S::CO:CFP #6* (harbouring multiple insertions) and *aba1-6 35S::CO:CFP #3* (single insertion). This choice was guided by two factors. First, amongst the lines analysed, these showed the earliest flowering (Fig. 5A). Secondly, they had similar levels of *CO:CFP* overexpression (Fig. 5B). In the T3 generation, *35S::CO:CFP(#6)* plants were significantly earlier flowering compared with wild-type Col-0 and *aba1-6 35S::CO:CFP* (#3), producing 20-25% fewer rosette leaves, respectively (Fig. 5D). In good agreement with the flowering time data observed in the T1 generation, reduced ABA production caused a delay in the floral transition in plants overexpressing *CO* (Fig. 5D and 5E). Such delay in flowering was well correlated with impaired *FT* transcriptional activation: At ZT12, *aba1-6*

35S::CO:CFP(#3) showed 7.5 fold reduction in *FT* transcript accumulation compared with *35S::CO:CFP(#6)*, despite showing similar levels of *CO:CFP* accumulation (Fig. 5E and 5F). Taken together these data continue to support my hypothesis that ABA is required for proper CO function on *FT* expression under inductive LD photoperiod.

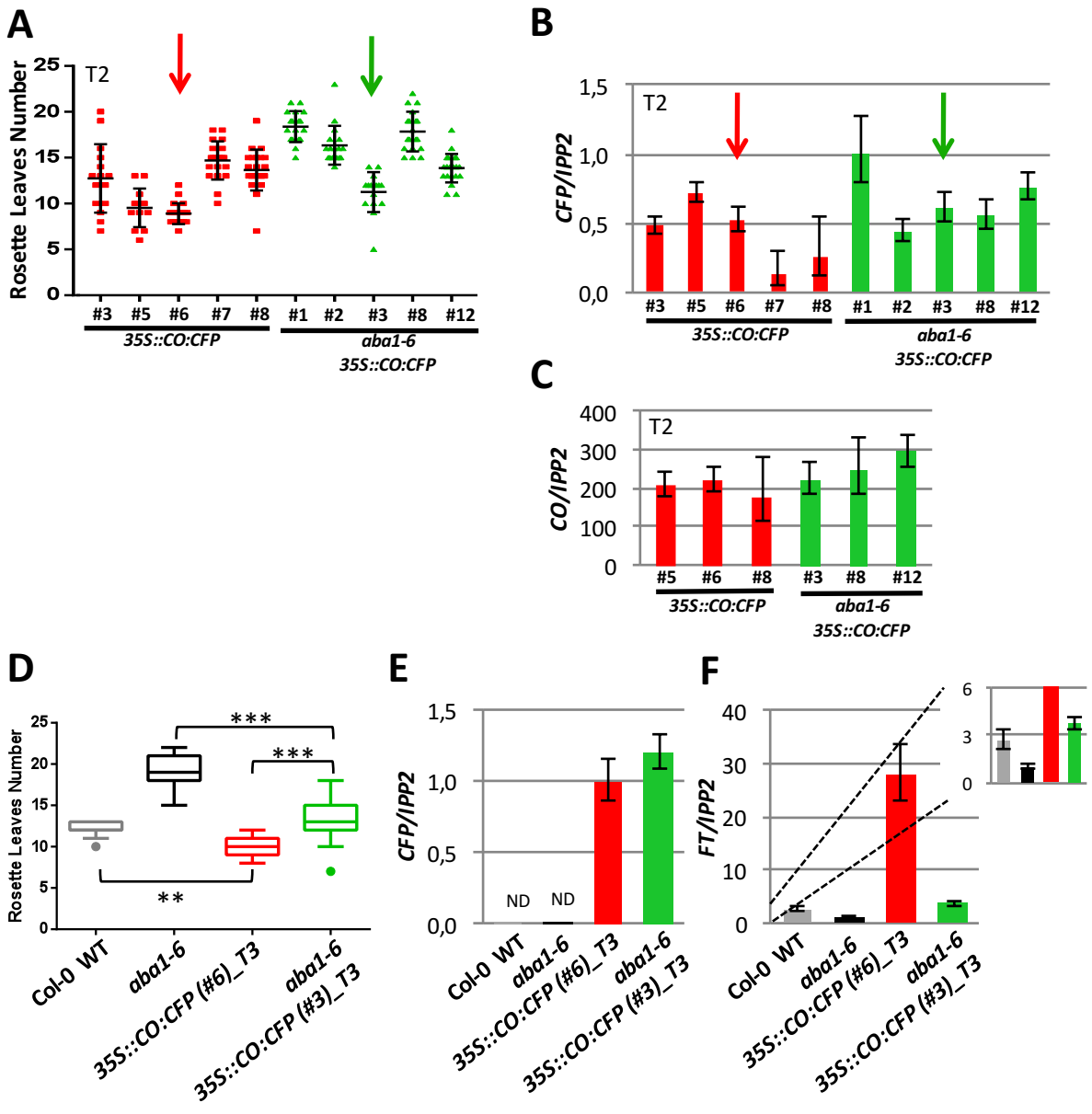

Fig. 5. Overexpression of *CO* produces heritable but not extreme early flowering phenotypes. (A) Scatter plot representing fluctuation in the flowering time, under LDs, of *35S::CO:CFP* T2 lines both in Col-0 and *aba1-6* backgrounds. Each dot represents a single Basta resistant T2 plant belonging to the segregating line indicated (each number represent a segregating T2 transgenic line). Error bars = SD, n=13-23. Red and green arrows indicate the T2 lines chosen for the further analysis in the T3 generation. **(B-C)** Real-Time qPCR of *CO:CFP* and *CO* transcript in two-week-old T2 seedlings grown under LDs following Basta selection at ZT12. Two Col-0 wild type plants were grown in parallel to evaluate transgene-derived *CO*

overexpression. Error bars are SD of two technical replicates. *IPP2* expression was used for normalization. **(B)** *CO:CFP* transcript levels were undetectable in wild-type non–transgenic plants. I thus used *aba1-6 35S::CO:CFP (#1)* for normalization. **(C)** Fold change variations of *CO* transcript levels of T2 transgenic plants relative to a wild type non-transgenic plant. **(D)** Tukey boxplot of the distribution of the number of rosette leaves of the indicated genotypes grown under LDs (n=17-20). *35S::CO:CFP (#6)* and *aba1-6 35S::CO:CFP (#3)* are non-segregating plants in T3 generation. Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.01 (**), ≤0.001 (***). **(E-F)** Real-Time qPCR of *CO:CFP* and *FT* transcripts in wild-type Col-0, *aba1-6* and T3 *35S::CO:CFP (#6)* and *aba1-6 35S::CO:CFP (#3)* 2-week-old seedlings grown under LDs and sampled at ZT12. Error bars = SD of two technical replicates. *IPP2* expression was used for normalization. **(E)** Values represent fold change variations relative to *35S::CO:CFP (#6)*. ND = not detectable **(F)** Values represent fold change variations relative to *aba1-6*. Inset shows lower level of *FT* transcript in *aba1-6* mutants compared to the wild type.

ABA signalling stimulates the function of CO upstream of *FT*

The canonical ABA signalling mediates the upregulation of the florigen genes (Riboni et al., 2016, 2013). The triple phosphatase mutant *hab1-1 abi1-2 abi2-2* has sensitised ABA signalling and an early flowering phenotype, which is correlated with increased levels of *FT* accumulation (Riboni et al., 2016, 2013). However, such increased levels of *FT* are not reflected in clear higher levels of *CO* under normal watering conditions (Riboni et al., 2016). Based on these data and the demonstrated effect of ABA in potentiating CO function (Fig. 4 and Fig. 5), I predicted that this background should be very sensitive to variations in CO protein accumulation. I therefore decided to transform *hab1-1 abi1-2 abi2-2* mutants (hereafter referred to as *3xabi*) with the previously described *35S::CO:CFP* construct and compared these plants to the wild type. In the T1 generation, more than half of the *3xabi 35S::CO:CFP* plants were extremely early flowering, producing between 4 and 6 leaves (Fig. 6A). Under similar conditions, the expression of *35S::CO:CFP* in the wild-type background produced such extreme early flowering phenotype in only 2 plants out of 28 T1 events. Furthermore, early flowering *3xabi 35S::CO:CFP* plants were semi-sterile, producing very few seeds. Because of this, *3xabi 35S::CO:CFP* lines were subsequently analysed as T2 segregating lines. Of two lines for which seeds were available, only one successfully germinated and after BASTA selection healthy seedlings we obtained. These were compared to T3 plants (subject to Basta selection) of *35S::CO:CFP (#6)*, representing the earliest and most stable line I managed to isolate. The phenotypic

differences between these transgenic lines are described in Fig. 6B and 6C. As compared with the wild-type background, overexpression of CO in *3xabi* plants conferred an extreme early flowering phenotype, which was accompanied by a strong reduction in plant size, effectively mimicking the previously described over-expression of *FT* or *CO* under the phloem companion cell-specific promoter *SUC2* (An et al. 2004; Jang et al. 2009 and below). I carried out expression analysis to compare the levels of accumulation of transgene-derived *CO* and to see how this was correlated with *FT* expression. Since I had very few plants of *3xabi 35S::CO:CFP (T2)*, and because of the small size of individual plants I decided to pool together the third expanded leaf from 5-7 *3xabi 35S::CO:CFP (T2)* plants at ZT12. A similar amount of tissues and using the same pooling strategy was collected from *35S::CO:CFP (#6)*. I did not observe differences in the accumulation of *CO:CFP* transcript between *3xabi 35S::CO:CFP (T2)* and *35S::CO:CFP (#6)*. However, *FT* expression was highly upregulated in *3xabi 35S::CO:CFP (T2)* as compared with the *35S::CO:CFP (#6)* (Fig. 6D and 6E). Although, as observed above, the *CaMV 35S* is not the most suitable promoter to overexpress *CO* and obtain extreme early flowering effects, these results strongly indicate that activated ABA signalling is sufficient to promote upregulation of *FT* under LDs, downstream of the transcriptional activation of *CO*.

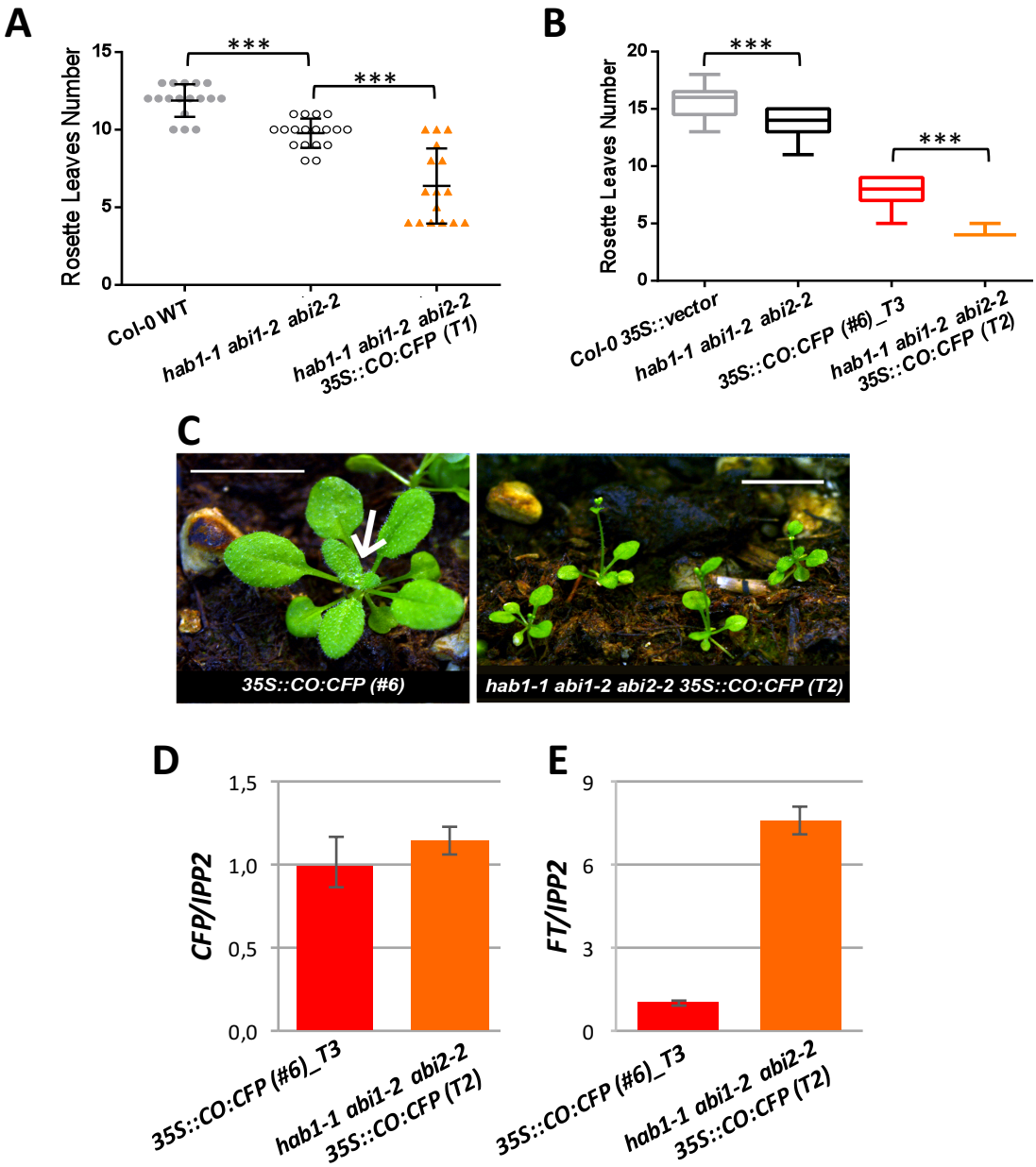

Fig. 6. The canonical ABA signaling promotes *CO* functions. (A-B) Scatter plot (A) and Tukey boxplot (B) of the distribution of rosette leaves number in the indicated genotypes grown under LDs. Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.001 (***). **(A)** Error bars = SD, n=16-17. **(B)** *35S::CO:CFP (#6)* is in the T3 generation. n=15, except for *3xabi 35S::CO:CFP (T2)* n=7. **(C)** Images of representative plants of *35S::CO:CFP* in the wild-type or *3xabi* backgrounds after 4 weeks from sowing. White arrow indicates a visible inflorescence. Scale bars= 1cm. **(D-E)** Real-Time qPCR of *CO:CFP* (D) and *FT* (E) transcripts in *3xabi 35S::CO:CFP (T2)* compared to *35S::CO:CFP (#6)*. The fourth leaves of 6-7 independent plants (included in the flowering time analysis shown in B) were harvested 20 days after sowing at ZT12 and pooled together. Error bars = SD of two technical replicates. *IPP2* expression was used for normalization.

ABA stimulates the function of CO in the phloem companion cells

As already discussed from other groups of the field, the ectopic expression of *CO* via the *35S* promoter in all the tissues of the plant might produce confounding effects, mainly as *CO* is only active in a particular cell–type, the phloem companion cells (An et al., 2004; S.-K. Kim et al., 2013; Song et al., 2012b). Unfortunately, the *35S* promoter might not ensure sufficiently high enough levels of *CO* transcript in the phloem companion cells. Based on these concerns and considering my difficulties in obtaining stable and early flowering *35S::CO:CFP* lines, I decided to generate another set of expression vectors where *CO* is under the control of the *SUC2* promoter. In this way, I could be able to confine CO overexpression in the phloem companion cells, and more precisely assess the role of ABA in the tissue where CO usually acts. Another potential problem of the *35S::CO:CFP* transgene might be the size and position of the tag at the C-terminal position. To exclude this possible artefact, I kept a GFP-like tag (CITRINE) fused at the C-terminus of *CO*. With the new construct, *SUC2::CO:CITRINE* (Fig. 7A), I transformed wild-type (Col-0) and two different ABA-deficient backgrounds (*aba1-6* and *aba2-1*) impaired at different steps of the ABA biosynthetic pathway (Finkelstein, 2013). A negative control was also used, consisting of the *SUC2::GW* construct (Fig. 7A). I then carried out a phenotypic study, by analysing the flowering time in more than 100 independent T1 plants for each transgenic line *SUC2::CO:CITRINE, aba2-1 SUC2::CO:CITRINE* and *aba1-6 SUC2::CO:CITRINE*, as well as vector-control transformations. In general, expression of *CO:CITRINE* in the phloem companion cells conferred a strong early flowering phenotype independent of the ABA content when these plants were compared with the *SUC2::GW* empty vector controls (*SUC2::vector*, Fig. 7B). Nevertheless, *aba1-6 SUC2::CO:CITRINE* and *aba2-1 SUC2::CO:CITRINE* plants showed a significant delay in flowering compared with *SUC2::CO:CITRINE* (Fig. 7B and 7C). Thus, unlike *35S::CO:CFP*, *SUC2::CO:CITRINE* conferred a much more dramatic early flowering phenotype in the T1 generation, ruling out

an impairment of CO function caused by the C-terminal fusion. In the light of this observation, I interpret the delay of flowering observed in the ABA-deficient T1 plants as extremely significant from the biological point of view (Fig. 7B). To easily illustrate this point, I grouped individual T1 plants based on phenotypic classes depending on the number of rosette leaves at bolting. The phenotypic distributions of *aba1-6 SUC2::CO:CITRINE* and *aba2-1 SUC2::CO:CITRINE* were highly overlapping and strongly shifted towards the higher number of leaves compared with the wild type background (which was highly skewed between 6-7 leaves) (Fig. 7D). To rule out a general effect of ABA on *SUC2* promoter activity, I carried out expression analysis on random independent T1 plants. Because of their particularly small size, I sampled the entire rosette of *SUC2::CO:CITRINE* plants (in the different genotypes), grown for 20 days under LDs, at ZT12. Expression from the *SUC2::CO:CITRINE* transgene produced similar levels of *CO:CITRINE* in the wild-type, *aba2-1* and *aba1-6* backgrounds (Fig. 7E). However, the strong *FT* upregulation (relative to Col-0 *SUC2::vector*) that I observed in *SUC2::CO:CITRINE* plants was nearly absent in the *aba2-1* and *aba1-6* backgrounds (Fig. 7E). As illustrated in *35S::CO:CFP* transgenic plants, I did not observe a clear correlation between *CO:CITRINE* expression and *FT* transcriptional activation. Rather, a clear trend was visible between *FT* transcript levels and the ABA status of the plants (Fig. 7E). Both the phenology and expression data obtained with the *SUC2::CO:CITRINE* construct confirmed what previously described for the *35S::CO:CFP* experiments. This allowed me to confidently conclude that ABA positively regulates flowering by promoting CO protein function.

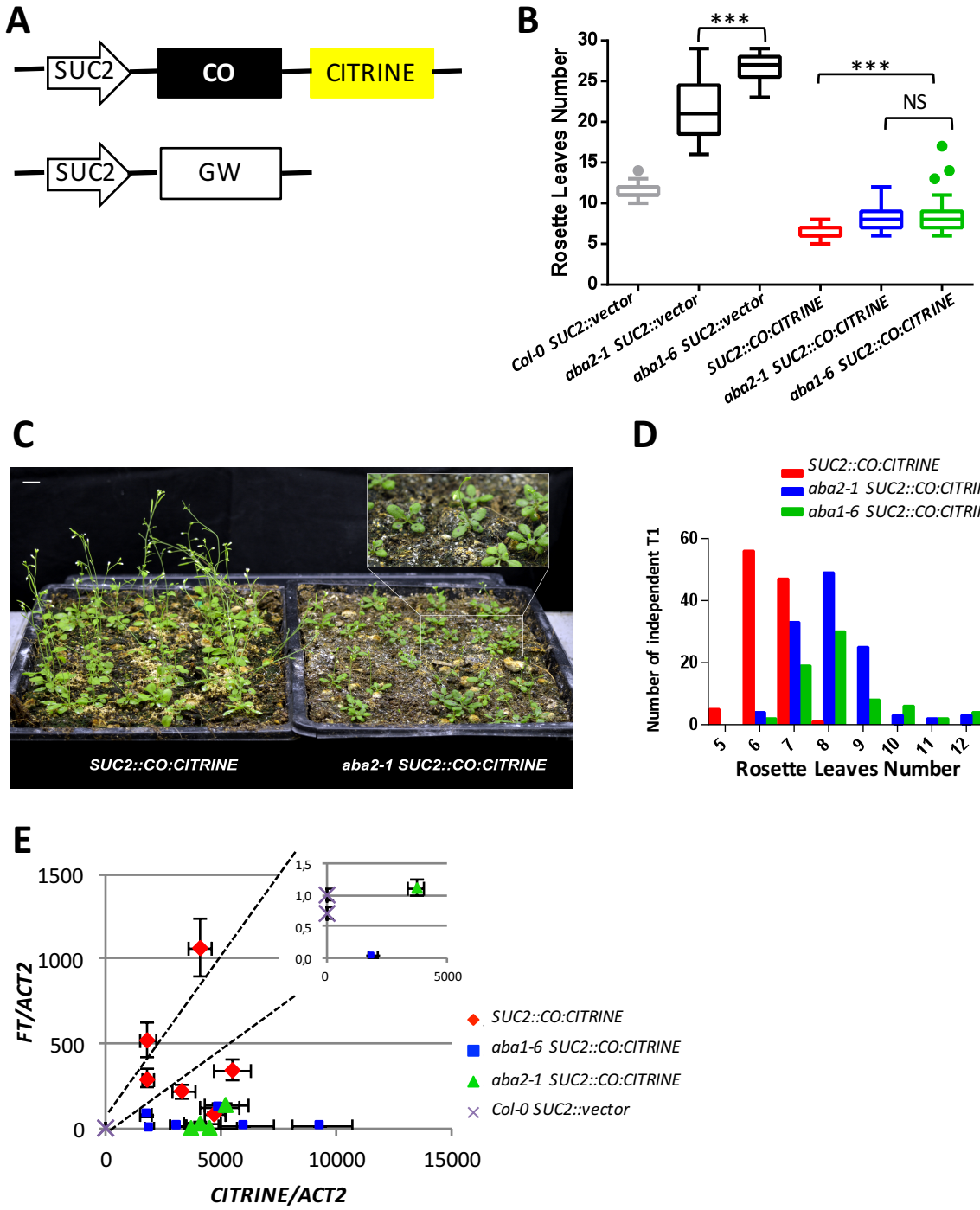

Fig. 7. ABA promotes CO functions in the phloem companion cells. (A) Diagram illustrating the main regulatory elements and tags in the constructs used for *Agrobacterium*-mediated transformation of *Arabidopsis*. **(B)** Tukey boxplot of the distribution of the rosette leaves numbers of T1 transgenic lines in the Col-0, *aba2-1* and *aba1-6* backgrounds grown under LDs. N = 16 for *SUC2::vector* controls. n= 70-100 for *SUC2::CO:CITRINE* transgene. Even if each plant analyzed represented independent insertion events, I preferred Tukey boxplot to scatter plot for graph clarity. Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.01 (**), ≤0.001 (***), >0.05 not significant (NS). **(C)** Image of Basta resistant T1 plants (included in the flowering time analysis shown in B). Inset shows *aba2-1 SUC2::CO:CITRINE* visibly later flowering compared to *SUC2::CO:CITRINE*. Bar scale = 1cm. **(D)** Number of T1 plants with the same rosette leaves number. Different colors indicate different genotypes (reported in the legend). **(E)** Scatter (XY) plot where *FT* transcript levels are compared to *CO:CITRINE*. The entire

rosette of 20-day-old T1 transgenic plants was harvested at ZT12, except for *Col-0 SUC2::vector* for which I sampled only expanded leaves. Values represent fold change variations relative to one wild-type T1 transformed with *SUC2::vector* (I chose this plant as normalizer, even if *CO:CITRINE* transcript was basically undetectable, for a better graphic clarity). Each dot corresponds to an independent T1 plant. Inset shows independent T1 plants with *FT* transcript levels lower than 1.5. Error bars represent the SD of two technical replicates. *IPP2* expression was used for normalization.

Protein studies in *N. bethamiana* suggest a role for ABA in the stabilization of CO

The early flowering observed under LDs compared to SDs depends on the light-mediated stabilisation of CO protein in the late afternoon (Song et al., 2012b; Valverde et al., 2004; Zuo et al., 2011). Based on the demonstration that ABA promotes CO function and since ABA affect the expression of *FT* and *TSF* at the end of a long day, when CO accumulates, (Riboni et al. 2013; Riboni et al. 2016; Unpublished data, Fig. 4-7), I wondered if the post-transcriptional effect of ABA might involve CO protein stabilisation.

Before testing this hypothesis in *Arabidopsis*, using CO-tagged isogenic lines in different ABA backgrounds, a rapid approach to verify if ABA had any roles on CO protein stabilization was through transient expression in *N. benthamiana*. I thus infiltrated leaves with *Agrobaterium tumefaciens* carrying the *35S::CO:CFP* construct (the same construct used to generate transgenic plants in *Arabidopsis*) or the *35S::GFP* construct which afforded a negative control (Fig. 8A). After three days under LDs, infiltrated leaves were harvested and immediately processed for ABA treatments and protein extraction. Because the level of expression of the transgene might change depending on the infiltrated leaves, I tried to randomize my sampling strategy as much as possible (see Materials and Methods). Leaf disks (n = 12-20 or more in the time course experiment) were incubated in petri dishes containing liquid MS supplemented with different concentrations of phyto-hormones or an appropriate diluent (mock). I then monitored the amount of immunologically detectable CO:CFP protein at different time points following the application of various phyto-hormones by western blot. Fig. 8B shows that CO:CFP protein was readily detectable at all time points in the absence of ABA in the medium, with some

variability occurring between the different time points. However, in the presence of exogenous ABA, CO:CFP levels were generally higher compared with the untreated control. Although this suggests that ABA reduces the endogenous turnover of CO, no obvious increase in CO:CFP protein level was observed as a result of longer incubations with ABA. In the same experiment I included a negative control *35S::GFP* construct to demonstrate that the stabilising effect of ABA on CO:CFP was specific for CO and not depending on the GFP-like tag (although some aminoacid vary between GFP and CFP) (Fig. 8C). These experiments were replicated twice and gave similar results, thereby a strong CO:CFP stabilization occurred within 15 minutes following ABA applications. To understand if the ABA-dependent CO stabilization was dose-dependent I repeated the experiment with different amounts of ABA in the medium (0.1-1-10-100 μM). I could confirm that ABA stabilised CO:CFP, but after 30 minutes of treatment I did not observe any dose dependency in the stabilization of CO:CFP (Fig. 8D). To test for the potential hormone-specificity of the observed effect I performed the same assay with other hormones involved in flowering time regulation and more specifically known to affect CO function at different levels, including gibberellic acid (GAs) and jasmonic acid (JA) (Hou et al., 2010; Porri et al., 2012; Xu et al., 2016; Zhai et al., 2015). Neither GA_4 10 μM nor JA 1 mM caused a stabilization of CO, suggesting that this ability was peculiar for the ABA hormone (Fig. 8D).

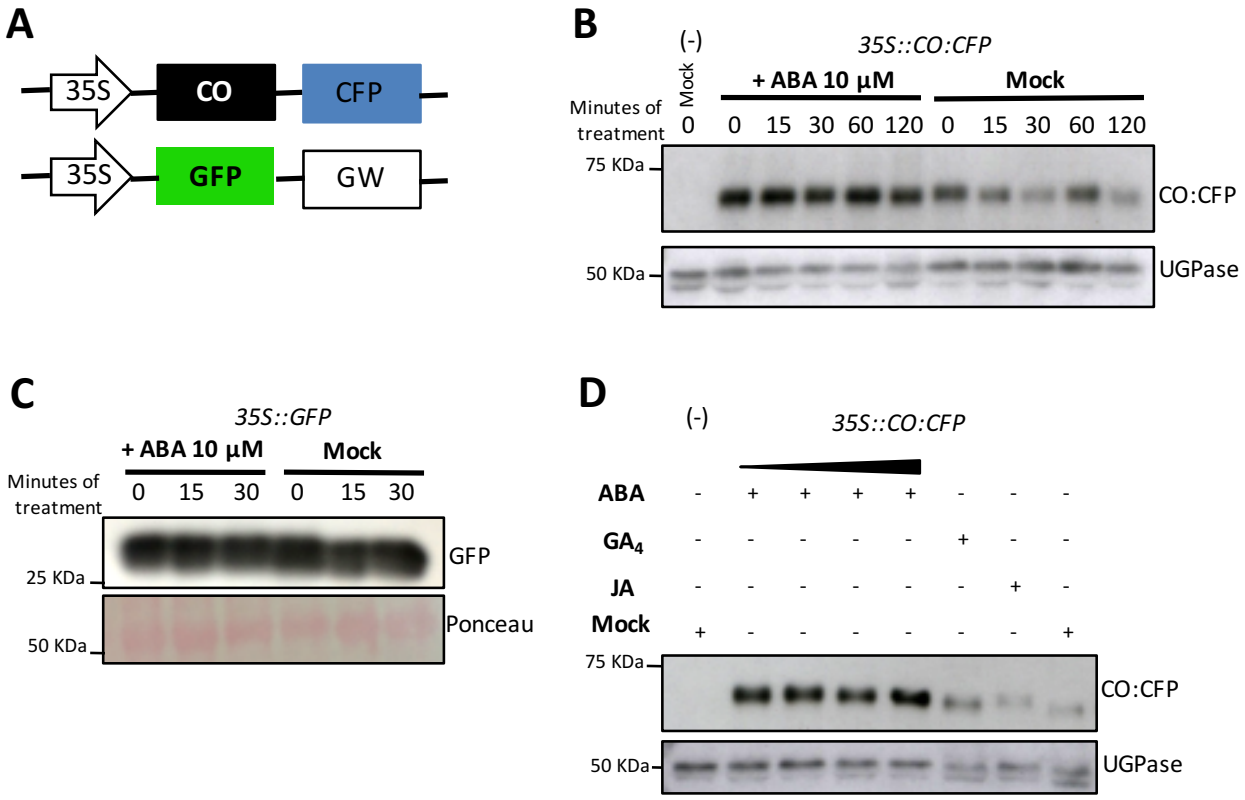

Fig. 8. ABA stabilizes CO when transiently expressed in *N. benthamiana* leaves. (A) Diagram illustrating the main regulatory elements and tags in the constructs used for *Agrobacterium*-mediated transient transformation of *N. benthamiana* **(B)** Western blot on total protein extracts of 15-20 disks from *Agrobacterium* – infiltrated leaves. Disks were made three days after infiltration and subjected to different ABA/Mock treatments for 0 to 120 minutes. The negative control (-) represents disks derived from non-infiltrated leaf submerged in mock solution before sampling. UGPase was used as loading control. Anti-GFP antibodies were used to detect CO:CFP. **(C)** *N. benthamiana* leaves were infiltrated with *Agrobacterium* containing *35S::GFP* vector. Western blot with Anti-GFP antibodies were used to detect GFP. Ponceau red staining of Rubisco Large subunit was used as loading control. **(D)** *N. benthamiana* leaves were infiltrated with *Agrobacterium* transformed with *35S::CO:CFP* vector. ABA, gibberellic acid (GA_4, 10 µM), jasmonic acid (JA, 1mM) and Mock solution were applied for 30 minutes. Black triangle shows increasing ABA concentrations (1-10-100-1000 µM). Table indicates presence "+" or absence "-" of the indicated hormone.

No evidence for an ABA-dependent stabilization of CO in *Arabidopsis*

To verify if ABA stabilises CO also in *Arabidopsis* I compared the abundance of CO in available T2 *35S::CO:CFP* lines. I tried different protein extraction protocols, both for nuclei isolation and total protein preparations. I could not obtain reliable results with none of the conditions tested because I always detected non-specific bands at the expected molecular mass of CO:CFP (70 KDa) also in the Col-0 *35S::vector* negative control. To exclude that the non-specific recognition was caused by the poor affinity of my antibody, I

tested different commercial anti-GFP antibodies but without any significant improvement. Because these antibodies could clearly detect CO:CFP in *N. benthamiana* experiments, I attributed these negative results to the low levels of CO:CFP protein being expressed in the transgenic lines. In Fig. 9 I illustrate a typical western blot result obtained with nuclei extracts with a commercial anti-Goat GFP antibody (Abcam). Even with the assumption that the CO:CFP signal is that indicated by the red arrow, I observed huge variability in its levels across the different transgenic lines (as observed for flowering time, Fig. 5A). If instead I consider the upper band as the specific signal, besides confirming the variability, I did not observe significant differences in the levels of CO:CFP accumulation between *35S::CO:CFP (#6)* and *aba1-6 35S::CO:CFP (#3)* (the most comparable lines, as discussed above).

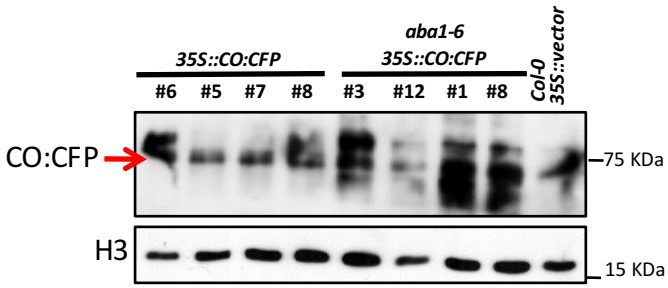

Fig. 9. CO:CFP detection in Arabidopsis transgenic plants. Western blot was performed on nuclear extracts of 11-day-old seedlings of different T2 lines *35S::CO:CFP* in Col-0 and *aba1-6* backgrounds grown under LDs. Col-0 plants transformed with the *35S::GW:CFP* empty vector (*Col-0 35S::vector*) were used as negative control. Anti-GFP antibodies detect a band of the predicted size of CO:CFP also in the negative control. Histone H3 detection was used as loading control.

Based on the problems encountered to obtain stable T3 *35S::CO:CFP* lines and because of the difficulties to detect CO:CFP in *Arabidopsis,* I decided to use a well-characterised and stable transgenic line where a tagged version of CO is expressed under the phloem specific promoter *SUC2* (*SUC2::HA:CO*, provided by the Coupland Lab) (Jang et al., 2009). This line was crossed into the *aba1-6* background with the aim to obtain an *aba1-6*

SUC2::HA:CO isogenic line. Plants of *SUC2::HA:CO* were extremely early flowering, similar to the *SUC2::CO:CITRINE* plants (Fig. 7B-C and 10A). *aba1-6 SUC2::HA:CO* plants were consistently and significantly later flowering than the parental *SUC2::HA:CO* line, producing on average 14.8 % +/- 5.4 more leaves (n = 3 independent experiments, 17 plants each) (Fig. 10A). At ZT12, *aba1-6 SUC2::HA:CO* had similar levels of *CO* transcript accumulation compared to the *SUC2::HA:CO* line but much reduced *FT* expression (approximately 50%) (Fig. 10B). These results are fully consistent with my previous observations about the role of ABA on CO activity in *35S::CO:CFP* and *SUC2::CO:CITRINE* transgenic lines. However, contradicting the results obtained in *N. benthamiana* transient assays, in *Arabidopsis* the levels of HA:CO nuclear protein were similar in *SUC2::HA:CO* and *aba1-6 SUC2::HA:CO* (Fig. 10C). To further test for the effect of ABA on CO protein stability I used the ABA root application protocol (Riboni et al., 2016), which mimics a drought escape signal and thus likely to promote CO function. I fed *SUC2::HA:CO* plants daily for 12 days with ABA at three different concentrations (0.25 -2.5 -25 μM) and on the 13th day I harvested tissues at ZT4 and ZT12 for nuclei isolation. After testing that this protocol could effectively trigger an upregulation of the florigen genes (Fig. 11A-H), I monitored the accumulation of nuclear HA:CO abundance. Also under these conditions I did not observe obvious changes in HA:CO at any of the concentration tested, either in the morning (when CO protein levels are low) or in the late afternoon (Fig. 10D).
Because these data were obtained in stable *CO* overexpressing *Arabidopsis* plants, my conclusions are that ABA promotes CO function without altering its stability and care should be exercised in interpreting the protein accumulation data obtained in experiments of transient expression in *N. benthamiana.*

ABA does not alter the phosphorylation status of CO

ABA signalling is based on a phosphorylation cascade raising the possibility that it might affect CO activity by modifying its phosphorylation status *in vivo* (Hubbard et al., 2010; Sarid-Krebs et al., 2015). In line with this hypothesis, the phosphorylated form of CO accumulates at dusk (Sarid-Krebs et al., 2015) when ABA-dependent signals are predicted to potentiate CO function and *FT* transcriptional activation. Although HA:CO protein was detected as a doublet, consistent with previous reports, the upper band (the phosphorylated one) was as intense as the lower band and I saw no alterations in the CO phosphorylation status between *aba1-6 SUC2::HA:CO* and *SUC2::HA:CO* (Fig. 10E). Similarly, exogenous ABA applications did not significantly affect the relative intensity of the two bands at any time point (Fig. 10F). These results argue against ABA being involved in modulating CO phosphorylation *in vivo*.

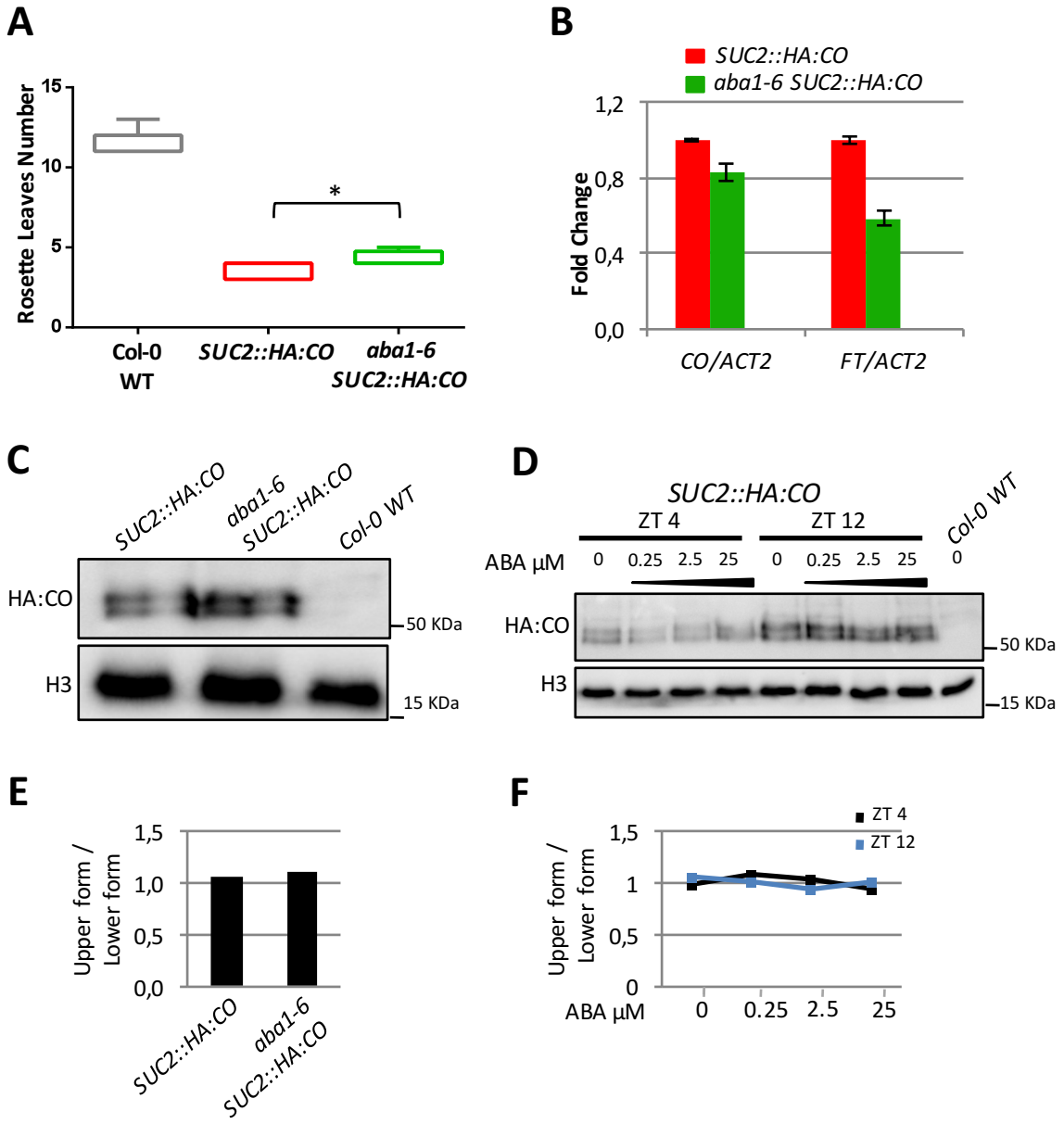

Fig. 10. ABA regulates CO function without altering its accumulation in Arabidopsis. (A) Tukey boxplot of the distribution of the rosette leaves numbers of *SUC2::HA:CO* isogenic lines and Col-0 wild-type plants grown under LDs (n = 17). Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤ 0.05 (*). **(B)** Real-Time qPCR of *CO* and *FT* transcripts in seedlings of *SUC2::HA:CO* lines grown for eleven days under LDs and harvested at ZT12. Error bars represent SD of two technical replicates. Values represent fold change variations relative to *SUC2::HA:CO*. *ACT2* expression was used for normalization. **(C-D)** HA:CO detection in nuclei extract by western blot. Anti-HA High Affinity antibodies were used to detect HA:CO and anti-H3 antibodies to detect H3 as loading control. Col-0 WT plants were used as negative control. **(C)** Plants were grown under LDs and 11-day-old seedlings were harvested at ZT12. **(D)** ABA soil applications to *SUC2::HA:CO* seedlings grown under LDs. A mock solution was used as a control. After germination, 12 days of ABA treatment were performed and seedlings were harvested at ZT4 and ZT12 on the 13th day. **(E-F)** Column (G) and dots (H) represent the ratio between the intensity of the upper and lower bands visible in (E) and (F), respectively. **(E)** Quantification of non-phosphorylated CO, relative to the upper band of *SUC2::HA:CO*. **(F)** Quantification of non-phosphorylated CO relative to the upper band of *SUC2::HA:CO*, ZT4, ABA 0 µM.

ABA promotes florigen expression without modifying the stability of GI protein

GI function has been described as key in drought escape response (Riboni et al., 2013). Our data also indicate that the drought-dependent upregulation of *FT* and *TSF* cannot occur if *GI* is absent, even when *CO* is highly expressed (Riboni et al., 2016). Furthermore, impaired ABA signalling blocks some aspects of GI function (*e.g.* the activation of *FT* and *TSF*) without altering *CO* expression (Riboni et al., 2016). These observations opened the new perspective that ABA activates *GI* signalling at the post-transcriptional level. The nature of this ABA-dependent posttranscriptional control on GI is however unknown. Similar to CO, GI protein abundance oscillates during the day, increasing during the day reaching a peak at ZT12 under LDs (David et al., 2006). The first hypothesis tested was if ABA may influence GI protein accumulation in *gi-2 35S::HA:GI* (David et al., 2006) using the ABA root application protocol (as described for *SUC2::HA:CO*) to induce ABA responses. The use of the *CaMV 35S* allowed me to separate confounding transcriptional effects from potential variations of HA:GI protein stability in response to ABA.

I initially tested whether continuous ABA applications could boost *FT* expression in wild-type plants or when *GI* is constitutively expressed. Since the treatment continued for several days, I followed the pattern of a well-known ABA marker (*RESPONSIVE TO ABA 18, RAB18)* (Lang et al., 1994; Lång and Palva, 1992; Mantyla et al., 1995) to identify which concentration was most effective in eliciting ABA responses. After 12 days of treatment *RAB18* expression increased when ABA was applied at a concentration of 2.5 μM, and sharply decreased at 25 μM (Fig. 11A and 11E). This suggests that prolonged ABA applications might eventually de-sensitise ABA signalling and shut down downstream transcriptional responses. As expected, the overexpression of *GI* caused an increase in the levels of *CO* transcript accumulation (between 2 and 3 times) compared with the wild type (Fig. 11B and 11F). However, I did not observe any further significant change in *CO* accumulation in response to any of the ABA concentration used (Fig. 11B and 11F). In *gi-2*

35S::HA:GI, ABA applications caused an increase in *FT* accumulation at the same concentration which was most effective for *RAB18* induction (2.5 μM) (Fig. 11G). Another florigen gene, *TSF*, was even more responsive to ABA soil applications, displaying a more pronounced increase compared to *FT* at lower concentrations of ABA (Fig. 11H). These data confirm that ABA activates the florigen genes downstream of *GI* transcriptional activation. Similar observations were made in the wild-type background where the *CO* transcriptional activation was not modified by exogenous ABA and *FT* expression was mildly increased at 0.25 μM ABA and fell at higher concentrations. The patter of *TSF* was highly variable and only partially followed that of *FT* (Fig. 11A-D).

As previously observed for *RAB18*, applications of ABA 25 μM caused downregulation of *FT* expression in both wild type and *gi-2 35S::HA:GI* backgrounds but, interestingly, not *CO* (Fig. 11A-H). This suggests that the florigen genes (particularly *FT*) are subject to a similar regulatory mechanism that controls ABA transcriptional responses, and this regulation does not affect *CO* transcript levels. Since plants of *gi-2 35S::HA:GI* were highly sensitive to exogenous ABA, I also monitored the pattern of expression of *RAB18*, *CO*, *FT* and *TSF* at ZT4. ABA-dependent responses were active at this time of the day (as inferred by *RAB18* expression), but much reduced compared to ZT12. *CO* expression was generally comparable to ZT12 and did not show variations in response to increasing levels of exogenous ABA. Transcript levels of *FT* and *TSF* were generally much lower compared to ZT12. ABA did not affect the expression of *FT* in the morning but did cause a small increase in *TSF* (ABA 2.5 μM treatment) (Fig. 11E-H). The fact that *RAB18* is more responsive to ABA at ZT12 compared to ZT4 (Fig. 11E), may suggest a broader interaction between ABA signalling and photo stimulated GI in the context of ABA transcriptional responses. Although it is well known that FKF1 stimulates GI function at this time of the day (Sawa et al., 2007), this data could suggest that GI is also sensitive to external ABA in a similar temporal window. However, at ZT12 no increased levels of

RAB18 expression were observed in *gi-2 35S::HA:GI* compared with the wild-type, suggesting that GI accumulation is not limiting in this process.

To test if ABA could promote GI function by increasing its stability, total proteins were isolated from *gi-2 35S::HA:GI* plants with the aim to monitor the levels of HA:GI protein at ZT12 (when I observed an ABA-dependent florigen upregulation) by western blot. I could easily detect the HA:GI band migrating at the predicted molecular mass as shown in Fig. 11I, but I did not observe significant changes in HA:GI protein levels upon any of the ABA treatments tested.

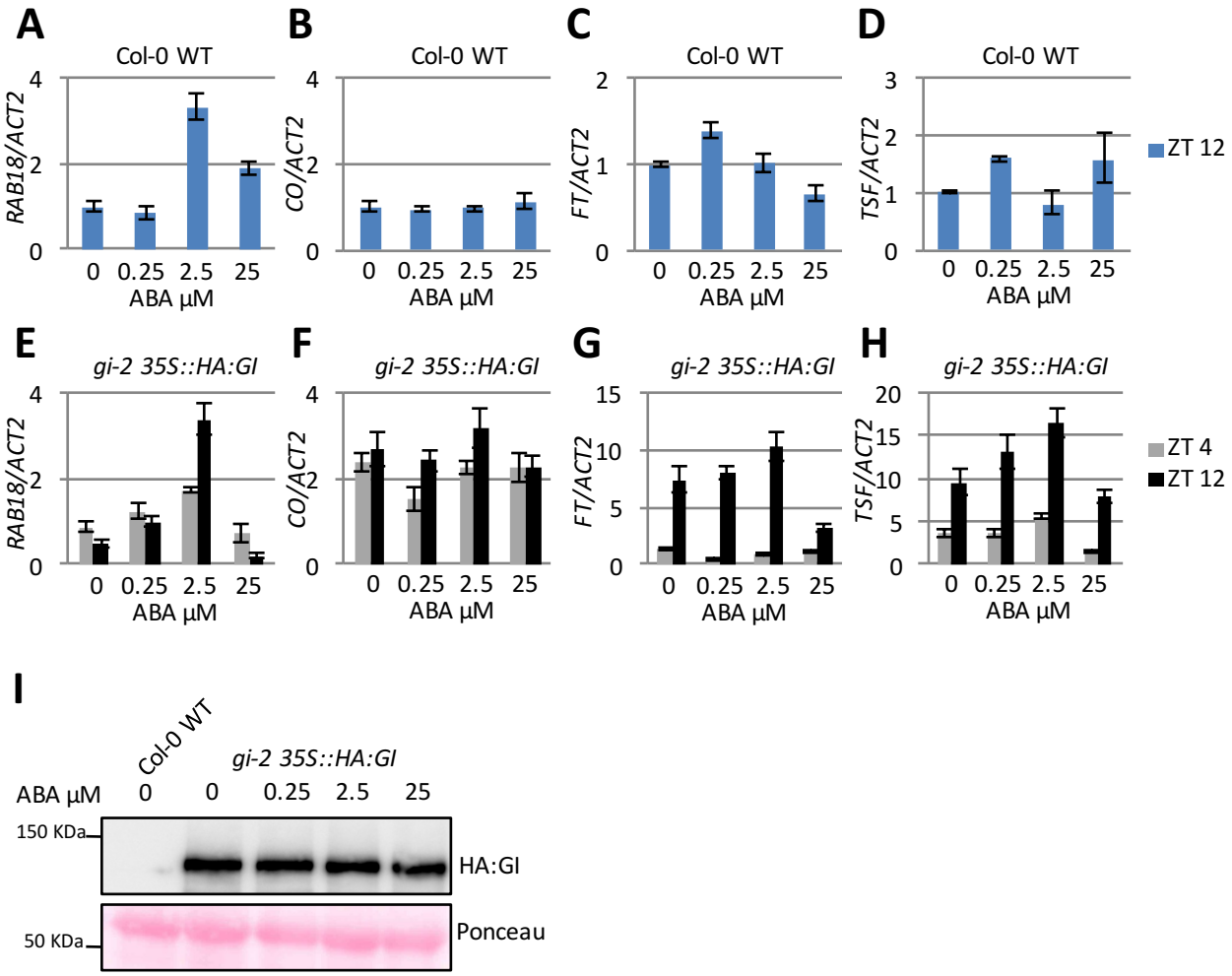

Fig. 11. ABA applications promote florigens expression without changes in GI protein accumulation. (A-H) Real-Time qPCR of *RAB18*, *CO*, *FT* and *TSF* transcripts in wild type (A-D) or *gi-2 35S::HA:*GI (E-H) seedlings grown under LDs and treated with ABA soil applications for 12 days. Seedlings were harvested at ZT4 and ZT12 on the 13th day. Mock solution was used as a control. Values represent fold change variations relative to Col-0 untreated control. Error bars represent SD of two technical replicates. *ACT2* expression was used for normalization. Similar results were obtained with *GI::GI:HA* transgenic plants **(I)** Detection of HA:GI at ZT12 in total protein extracts of *gi-2 35S::HA:GI* seedlings grown and treated as previously described. Col-

0 wild type plants were used as negative control. Ponceau red staining of Rubisco large subunit afforded a loading control. No changes in GI:HA accumulation were observed in *GI::GI:HA* transgenic plants.

To further support these results, I compared the amount of GI:HA protein in two isogenic lines obtained by crossing a publicly available *gi-2 GI::GI:HA* with *aba1-6*. Three *aba1-6 gi-2 GI::GI:HA* lines (#2, #11, #16) were isolated in the fourth generation as detailed in Materials and Methods. After 12 days under LDs, I collected tissues for both *GI* expression studies and protein analysis. Although the transgenic *gi-2 GI::GI:HA* plants showed higher levels of *GI* transcript compared with the wild type (which might be due to the contribution of the *gi-2*-derived endogenous transcript or to a more active *GI* promoter cloned in the transgene), I observed similar high levels of *GI* transcript in the three *aba1-6 gi-2 GI::GI:HA* lines (Fig. 12A) compared with *gi-2 GI::GI:HA*. The levels of GI:HA protein were also unchanged in *aba1-6 gi-2 GI::GI:HA* lines as compared with *gi-2 GI::GI:HA* (Fig. 12B), indicating that impaired ABA production does not affect the stability of GI in the late afternoon.

gi-2 GI::GI:HA plants flowered earlier compared with the wild type, a phenotype presumably derived from imprecise incorporation of all the regulatory elements required for *GI* transcriptional regulation in the original promoter construct (Kim et al., 2007). However, despite no changes in GI protein accumulation were apparent in *aba1-6 gi-2 GI::GI:HA* across independent experiments, I clearly observed a consistent and significant suppression of the early flowering phenotype conferred by *GI::GI:HA* in *aba1-6 gi-2 GI::GI:HA* lines (Fig. 12C). This result underscores the post-transcriptional effect of ABA on GI protein signalling in the context of the floral activation, although this post-transcriptional effect cannot be explained in terms of variations in GI protein accumulation.

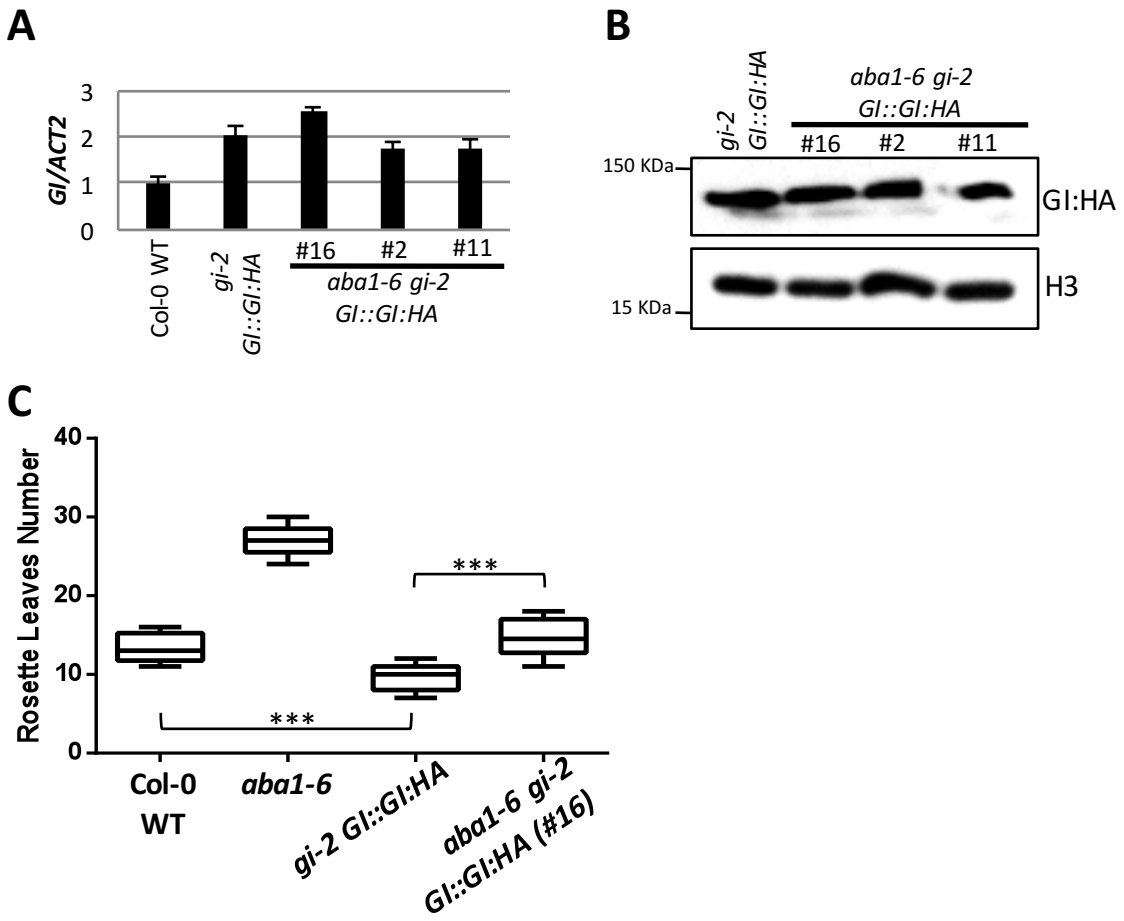

Fig. 12. Lack of ABA does not alter GI protein accumulation. (A) Real-Time qPCR of *GI* transcript in 12-day-old seedlings grown under LDs and harvested at ZT12. *gi-2 GI::GI:HA* and *aba1-6 gi-2 GI::GI:HA* are isogenic lines obtained by genetic crossing. Values represent fold change variations relative to Col-0 WT. Error bars represent SD of two technical replicates. *ACT2* expression was used for normalization. **(B)** Western blot detection of GI:HA in total protein extracts of 12-day-old seedlings grown under LDs and harvested at ZT12. Monoclonal Anti-HA antibodies were used to detect GI:HA and anti-H3 antibodies to detect H3 as loading control. **(C)** Tukey boxplot represents the distribution of the rosette leaves numbers of indicated genotypes grown under LDs (n= 15-17). Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.001 (***).

Role of CO in recruiting GI and FKF1 in nuclear speckles

My results demonstrate that ABA positively controls GI and CO signalling to activate drought escape, but does not affect their protein accumulations. Thus, I started to test other models to explain the molecular basis of drought escape. For example, both GI and CO directly bind to the *FT* promoter and contribute to its transcriptional activation (Sawa and Kay, 2011; Tiwari et al., 2010). Could it be that GI affects how CO protein binds to the *FT* chromatin? does ABA affect the recruitment of these protein components onto the

promoter of *FT*? I started to address these questions by generating fluorescently-labelled versions of CO and GI and use these constructs in transient expression assays in *N. benthamiana*. I then deployed laser scanning confocal microscopy to visualize CO and GI localisation in plant nuclei. Each image reported in Fig. 13 is representative of three independent experiments in which I monitored 4/5 different cells. I first analysed GI and CO localisations in single transformation events. CO protein was localised in very small nuclear bodies, evenly distributed in the nucleus. This punctuate pattern unlikely derived from an artefact of the fusion protein (citrine) since similar observations were made with different fluorescently-tagged versions of CO, indicating that the signal localisation depended on the protein rather than the tag used (Fig. 13A). Also GI:cherry was localised in nuclear bodies. However, these were larger and fewer compared to those observed in CO transformations (Fig. 13A and 13B). In double infiltration experiments I aimed to verify how CO and GI might localise in plant nuclei. I compared the fluorescent signals derived from cherry and CFP because their spectral emissions do not overlap, thus excluding mis-interpretations. I also used a positive control, FKF1:cherry, to validate my experimental settings since FKF1 is a well-established interactor of GI (Sawa et al., 2007). In FKF1:cherry single infiltrations I observed a fluorescent signal which was evenly distributed in the nucleoplasm, with poorly defined speckles which were not consistently observed in all the cells analysed (Fig. 13C). However, in combination with GI, FKF1 totally changed its localisation by relocating in the pattern of GI, suggesting that the re-localisation of FKF1 was driven by protein-protein interactions (Fig. 13F). When I co-infiltrated leaves with *35S::GI:cherry* and *35S::CO:CFP*, GI re-localized in the pattern of distribution of CO as the two fluorescent signals overlapped into the CO-specific nuclear bodies (Fig. 13D). FKF1:cherry also co-localised with CO:CFP even if FKF1 did not change its nuclear distribution so dramatically as when in presence of GI (Fig. 13E and 13F). I excluded that CO:CFP could cause re-localisation of every co-expressed protein

because when I co-infiltrated leaves with *35S::CO:CFP* and *35S::TZP:cherry* (Kaiserli et al., 2015) the two proteins maintained their original pattern of distribution in the nuclei with no or very poor overlap of their fluorescence signals (Fig. 13G). As my results indicate that CO can trigger re-localization of GI and (to a lesser extent FKF1) to specific nuclear bodies, I wanted to test whether all these proteins could be found in the same speckles at the same time. Co-expression of GI:cherry, FKF1:CFP and CO:CITRINE showed that the different fluorescent signals overlapped in most (but not all) of the nuclear bodies which were similar in size and distribution to the ones characteristic of CO:CITRINE (Fig. 13H). Interestingly also the pattern of FKF1 protein became much sharper in nuclear bodies, suggesting a stronger re-localization from the nucleosol to the speckles, possibly mediated by the presence of GI. Although the precise nature and significance of the CO speckles remain to be investigated, my confocal microscopy data suggest that CO recruits GI to precise locations, perhaps through direct protein-protein interactions. FKF1, through its tight interaction with GI is also tethered at these locations where a more stable protein complex with CO might be formed. It remains to be evaluated if ABA has a role in this postulated complex formation and CO-mediated recruitments. However, with the genetic tools I have generated (e.g. *SUC2:CO:CITRINE*) I shall be able to confirm these results directly in Arabidopsis.

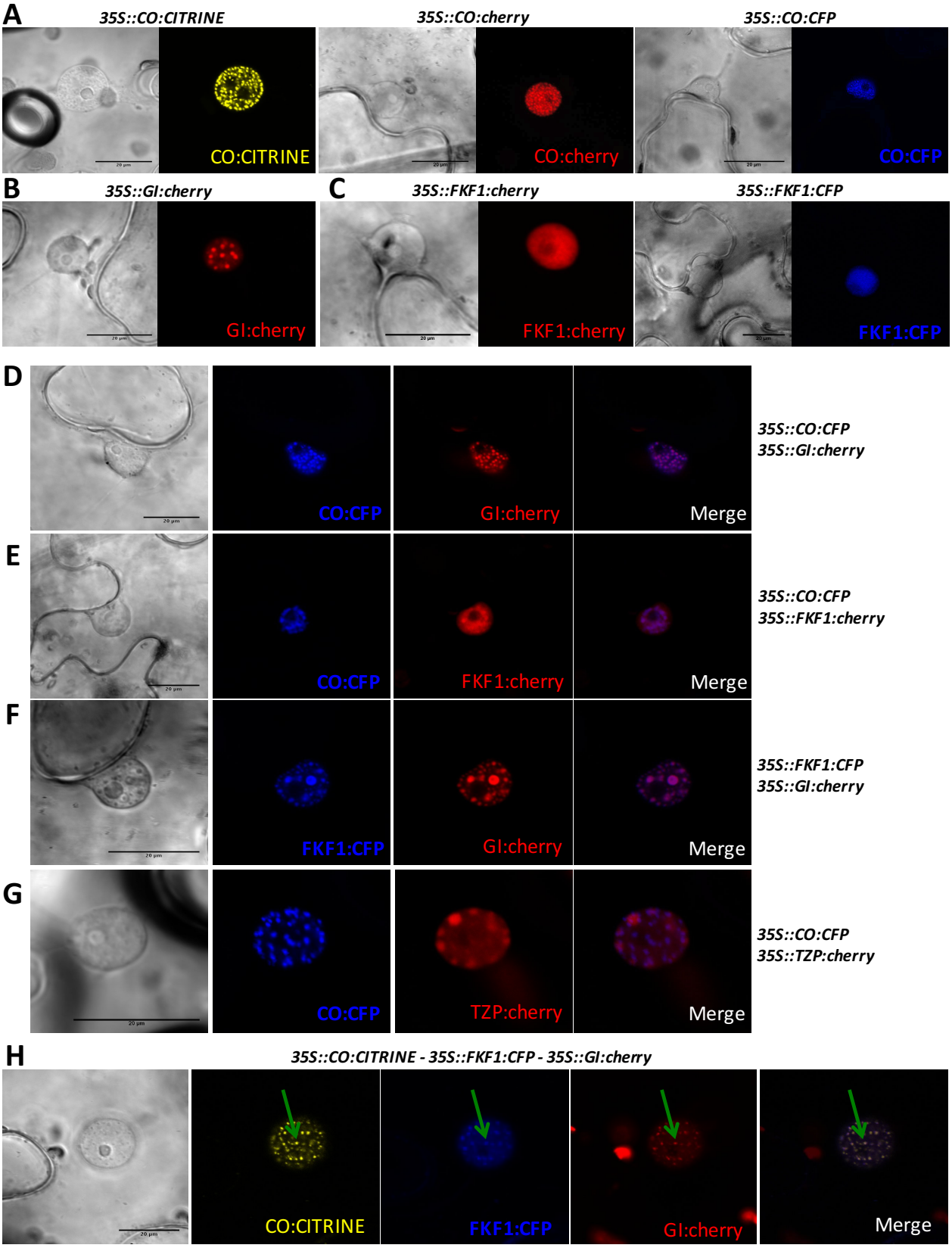

Fig. 13. CO recruits GI and FKF1 in nuclear bodies. (A-C) Representative images of bright field and fluorescence signals in nuclei of *N. benthamiana* leaves transiently transformed with 35S::CO:*CITRINE*, *35S::CO:cherry*, *35S::CO:CFP* (A), *35::GI:cherry* (B), *35S::FKF1:cherry*, *35S::FKF1:CFP* (C). Bars scale= 20 µm. **(D-H)** Representative images of bright field, single channel fluorescence signals and merge of fluorescence in co-transformed nuclei of *N. benthamiana* leaves. Combinations of constructs used for the transient expression are listed in the figure. Bars scale= 20 µm.

Future perspective: ChIP and CoIP experiments will help decipher the role of ABA in modulating GI and CO function

Recent data indicate that nuclear bodies may represent chromatin regions of intense transcriptional activity in flowering time regulation (Kaiserli et al., 2015). Because unlike previously thought (Wigge et al., 2005), CO protein appears to regulate a much larger number of target genes (Gnesutta et al., 2017), my data may point to a possible role of CO in recruiting higher order complexes at different genomic locations to initiate transcriptional events. The role of ABA in this context could be analysed using isogenic lines *gi-2 GI::GI:HA* and *aba1-6 gi-2 GI::GI:HA* with which one can investigate the occupancy of GI protein at the *FT* promoter according to varying levels of endogenous ABA. I propose similar experimental approaches for CO protein, in the different ABA-related backgrounds. However, due to time constraints I could not take these ideas any further.

Since GI and CO physically interact in Yeast and *in Planta* (Song et al., 2014a) one might ask whether this complex formation is ABA dependent. Because a CO antibody is not available to us, and because the generation of double transgenic plants with tagged CO and GI would be time consuming, I started to explore alternative ways to understand if ABA modulates the GI-CO interaction. I optimised a heterologous FLAG:CO production system using *in vitro* transcription/translation. I tested whether the heterologous CO could be subject to proteasome-dependent degradation in a cell-free assay (Valverde et al., 2004). When FLAG:CO was incubated with total protein extracts of wild type plants without the MG132 proteasome inhibitor, the FLAG:CO signal decreased over time, with a half-life of 15 minutes of incubation (+PIC, Fig. 14A and 14B). In contrast, in the presence of MG132, FLAG:CO was stable showing no obvious decay for two hours after incubation (+MG132, Fig. 14A and 14B). In the future I plan to use this protein for incubation with affinity purified GI:HA protein derived from wild-type or *aba1-6* backgrounds in CoIP

experiments. My work thus contributes to address these points *in vivo* by providing several useful genetic tools for both confocal imaging and molecular studies.

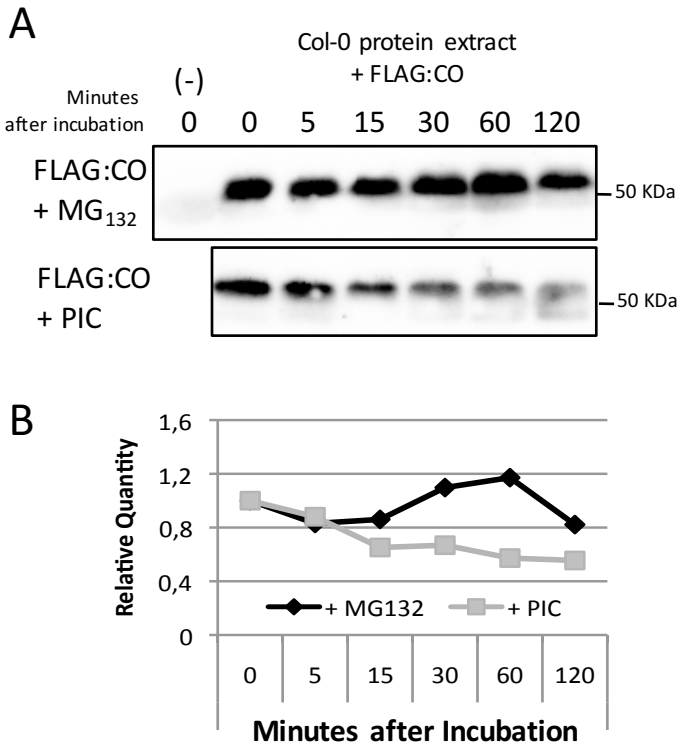

Fig. 14. Heterologous FLAG:CO is subject to proteasome-dependent degradation. (A) Western blot detection of FLAG:CO produced with the TNT transcription/translation system and incubated with total protein extracts from Col-0 wild-type plants for a cell free degradation assay. Aliquots of the reaction were sampled at indicated time points. The negative control (-) represents the total protein extract without FLAG:CO. MG132 was used as proteasome inhibitor; Protease inhibitor cocktail (PIC) was used as a control for nonspecific degradation. **(B)** Quantification performed with Image Lab and relative to T0 (0 minutes) of each experimental condition.

3.2 Side Project

Role of the ABA-related *bZIPs* in flowering

ABA signalling is connected to transcriptional events through a class of bZIP transcription factors (Cutler et al., 2010; Yoshida et al., 2014). Interestingly, the ABA-related bZIPs are structurally similar to FLOWERING D (FD), the main interactor of FT and a key flowering regulator in the SAM (Abe et al., 2005). While I was generating genetic crosses between ABA deficient and the different photoperiodic signalling mutants (Riboni et al., 2016) I included in my analysis also the *fd* mutants. Unexpectedly, *aba1-6 fd-4* double mutants produced a strong delay of flowering compared with their parental lines (Fig. 15A). In one interpretation, the effect might derive from ABA acting through *FT* thereby the mutant phenotype might depend on combined decreased *FT* levels (as a result of reduced ABA accumulation) and loss of FD function in the apex. However, previous data showed that combinations of *aba1* or *abi1-1* with *soc1* (an important gene acting in the SAM, partially downstream of FD) caused only a small delay of flowering compared to *soc1* (Riboni et al., 2016, 2013). Therefore, another interpretation would be that, in the absence of FD, a reduction in ABA might expose other FD-like functions that require ABA. FD is a Basic Leucine Zipper Domain (bZIP) transcription factor, in a side project I thus started to characterise additional mutants in the ABA-related bZIP factors. I focused my genetic analysis on *ABRE-BINDING FACTORS* (*ABFs*) and *ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3* (*AREB3*) that belong to the bZIPs clade A as FD (Choi et al., 2000). Since the ABFs are functionally redundant (Yoshida et al., 2014), I generated double and triple mutants before analysing the flowering time under LDs. Mutants of *abf3 abf4* did not show clear flowering time defects compared to the wild type (Fig. 15B). However, the triple mutant *abf1 abf3 abf4* had a significantly delayed flowering compared with the wild type and also to the double mutant of *abf3 abf4*. Since triple mutants of *abf1 abf3 abf4* are late

flowering, an interesting point would be to understand if the positive role of ABA on flowering depends on the ABFs. However, previous reports have shown that the ABFs positively regulate flowering through the transcriptional activation of *CO* (Yoshida et al., 2014). Thus, it is unlikely that ABA affects CO function through the ABFs. Additional molecular work aimed at deciphering the role of the *ABF* genes in flowering is ongoing in collaboration with the G. Couplands' lab (MPI, Cologne).

As the closest related bZIPs to ABFs, *AREB3* and its homologue *ENHANCED EM LEVEL* (*EEL*) (Choi et al., 2000) may have similar positive effects on flowering. In contrast to my expectations, in independent experiments *areb3* knock out mutants produced slightly fewer rosette leaves compared with the wild type (Fig. 15A and 15B). These data suggest that bZIPs have separate functions in the regulation of the floral transition. I generated the double mutant *areb3 eel* and preliminary results argue against a redundant role between these genes, as I observed no further acceleration of flowering time compared with *areb3* (data not shown). To further extend this genetic analysis, I crossed *areb3* and *aba1-6* plants to obtain the double mutants of *aba1-6 areb3*. Plants of *aba1-6 areb3* generated a dramatic late flowering phenotype compared to *aba1-6*, effectively mimicking the phenotype of *aba1-6 fd* double mutants (Fig. 15A). More molecular work is required to interpret this phenotype, as AREB3 might have FD-like function in the SAM, but this molecular activity is only apparent under low ABA conditions. Another hypothesis is that AREB3 is required for florigen genes expression, although this does not account for the mild early flowering phenotype of *areb3* mutants. In this perspective, the analysis of the site of expression of the different bZIPs and their mis-expression in plants will be crucial to distinguish between the two models.

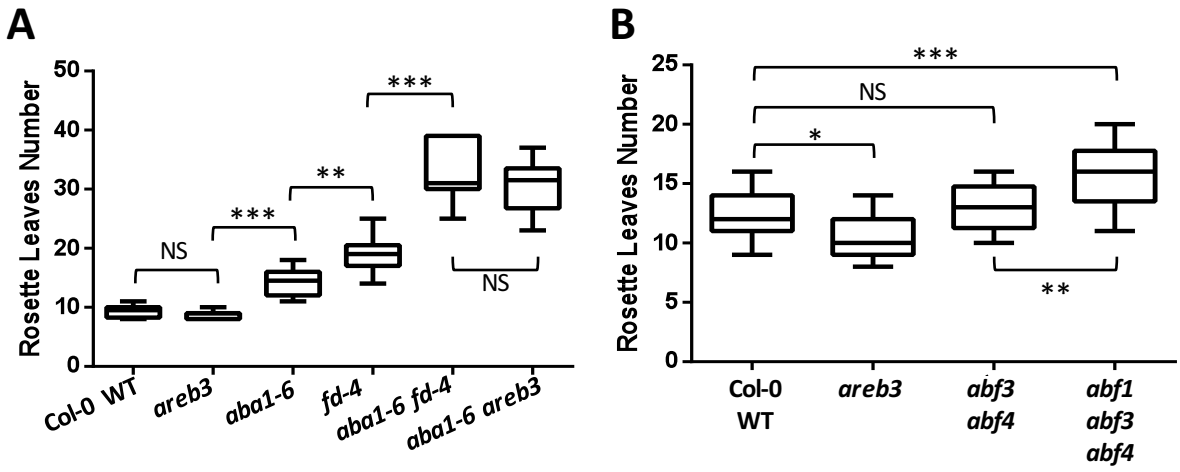

Fig. 15. ABA-related bZIP are involved in flowering time control under LDs. (A-B) Tukey boxplots of the distribution of the rosette leaves numbers of the wild type (Col-0) and ABA-related bZIP mutants grown under LDs (n=15-17 plants for each genotype). Multiple comparisons were performed with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.05 (*), ≤0.01 (**), ≤0.001 (***), >0.05 not significant (NS).

3.3 Discussion and Future Perspectives

In *Arabidopsis* ABA-stimulated acceleration of flowering depends on upregulation of the florigen genes at the end of a long day. My published data demonstrate that this event requires functional GI and CO. ABA regulates GI and CO functions mainly at the post-transcriptional levels, but this does not involve changes in their protein accumulation. My work thus offers new ground for investigating possible molecular mechanisms that could explain the ABA-dependent level of regulation of photoperiodic flowering.

Genetic interaction between ABA and photoperiodic flowering

Attempts were made to pinpoint a molecular interaction between ABA and photoperiodic flowering through genetic crossing of ABA-deficient and photoreceptors mutants. I detected an epistatic effect of *phyB* on ABA-defective mutants in flowering, which could suggest an interaction between ABA and red light signalling (Fig. 3A). In this context, ABA might affect CO function via negative modulation of PhyB function. The early flowering phenotype of *phyB* mutants depends on increased accumulation of CO protein in the morning (Valverde et al., 2004), pointing to a role for ABA action in this temporal window. However, CO:HA accumulation in response to ABA applications revealed no evident changes at ZT4 (Fig. 10D), thus weakening this hypothesis. Interestingly, besides mediating RL signals, PhyB (along with other phytochromes) has been recently described as a thermosensor (Jung et al., 2016; Legris et al., 2016). Mutants of PhyB show a constitutive warm-temperature response, which may contribute to their early flowering phenotype. Clearly more work is needed to establish how ABA and PhyB signalling interact and whether this epistatic interaction underlies a common target or rather derived from PhyB deregulating several flowering pathways during the day (light and temperature-related), which could mask the ABA effects on flowering.

ABA promotes CO functions in the vascular tissue

My attempts to over-express CO under the *35S* promoter revealed that although *35S::CO:CFP* could not confer a robust early flowering phenotype in transgenic plants, this phenotype was much more attenuated in the *aba1* background (Fig. 4B and Fig. 5A, 5D). A clear trend of *FT* expression emerged when comparing *35S::CO:CFP* transgenic lines in the wild type or ABA-deficient backgrounds, which I could not clearly relate to variations in *CO* transcript accumulations (Fig. 4C-E and Fig. 5E, 5F). Thus, the delay in flowering in *aba1-6 35S::CO:CFP* compared to *35S::CO:CFP* could depend on impaired ability of CO to upregulate *FT,* which strongly indicates that CO function requires ABA production (Fig. 4C-E and Fig. 5E,5F).

The mild and variable early flowering phenotype observed across different *35S::CO:CFP* transgenic lines opens a question about the activity of *CO* when over-expressed in tissues others than the phloem companion cells. Such high variability in flowering time may have different explanations: first, CO could have negative/interfering effects on the floral transition outside of its tissue of competence; secondly, the *35S* promoter might not ensure sufficiently high enough levels of *CO* transcript in the phloem companion cells. Previous mis-expression data do not support the first hypothesis, but do suggest that the site of *CO* expression is key for its function (An et al., 2004). Interestingly, while *35S::CO:CFP* conferred an extremely variable flowering phenotype in wild-type plants, in *3xabi* mutants it caused a highly stable and extreme early flowering (Fig. 6A-C). Hypersensitivity to ABA thus alleviates the above-discussed problems associated with ectopic expression with the *35S*. Since ABA production and signalling is highly active in the phloem companion cells of the leaf (Endo et al., 2008; Kuromori et al., 2014; Mustilli et al., 2002), low levels of *CO* transcript in the phloem companion cells might be compensated by post-transcriptional effects on CO protein as a result of enhanced ABA signalling in those cell-types. In support of the important role of ABA in affecting CO

function in the phloem, the robust early flowering phenotype conferred by *SUC2:CO:CITRINE* expression was significantly attenuated in independent ABA-deficient backgrounds, which was reflected in severely diminished levels of *FT* transcript accumulation (Fig. 7). The results obtained with *aba1-6 SUC2::HA:CO* and *SUC2::HA:CO* isogenic lines further demonstrate the promotive effect of ABA on CO function in the phloem companion cells of plants that differ only in the ABA content (and without confounding background effects) (Fig. 10A-D).

ABA-dependent activation of photoperiodic signalling unlikely involves CO protein stabilization

Initial transient expression experiments in *N. benthamiana* have led me to hypothesise a direct role for ABA on CO protein stability (Fig. 8), which I excluded afterwards in *Arabidopsis* (Fig. 10C and 10D). These contrasting results are probably ascribed to the exogenous ABA treatment performed on *N. benthamiana* leaves. Because CO:CFP expression occurs in all the Agro-transformed cells, it is possible that ABA causes non-specific changes in its stability. While it is interesting to note that the observed effects were ABA-specific, their precise cause was not investigated further. As discussed earlier, the site of CO expression may affect its function and possibly its mode of accumulation. For example, *35S::3HA:CO* and *SUC2::HA:CO* transgenic lines do not show a complete overlap in their pattern of accumulation of CO protein during the day (Hayama et al., 2017; Song et al., 2012b). Moreover, mutants of *fkf1* impair CO protein accumulation much more clearly in *35S::3HA:CO* lines compared with *SUC2::HA:CO* suggesting that tissue specificity affects key aspects of CO protein post-transcriptional regulation. These considerations led me to optimise conditions to monitor variations in CO protein levels in *Arabidopsis* where, unlike *N. benthamiana*, ABA does not affect its abundance (Fig. 10C and 10D). I finally found the right combination of nuclei extraction protocol/antibody to

detect CO. The newly developed *SUC2::CO:CITRINE* lines in the wild-type and ABA-related backgrounds will allow me to confirm that ABA does not affect CO protein abundance or phosphorylation status in many more independent transgenic lines other than *SUC2::HA:CO* (since I started to notice a high degree of co-suppression of *CO* expression in *aba1-6 SUC2::HA:CO* lines in the latest generation) (Fig. 10C-F).

Alternative scenarios for the ABA-dependent activation of photoperiodic signalling

My cumulated results point to other molecular mechanisms through which ABA affects photoperiodic flowering upstream of *FT*. Because drought stress cannot stimulate the transcriptional activation of *FT* in the absence of functional CO, CO must be a limiting factor in the drought/ABA-dependent activation of *FT* (Riboni et al., 2016). The fact that under SDs neither drought stress nor hyper activation of ABA signalling (e.g. the *3xabi* mutant background) is sufficient to re-activate *FT* expression, suggests that ABA must act after or in coincidence with photoperiod-stimulated *CO* expression. I cannot ignore that ABA contributes to the transcriptional activation of *CO*, but my data also corroborate a posttranscriptional effect (Riboni et al., 2016).

In Arabidopsis CO is the key activator of *FT* (Suarez-Lopez et al., 2001); CO directly binds DNA at the CO-responsive elements (CORE) in the *FT* promoter (Cao et al., 2014; Gnesutta et al., 2017; Tiwari et al., 2010); additionally, CO recruits several other proteins (*ASYMETTRIC LEAVES 1*, *AS1*, and members of the *NUCLEAR FACTOR Y* family, *NF-Y*) that collectively act as trans-acting factors, boosting *FT* transcriptional activation (Ben-Naim et al., 2006; Cao et al., 2014; Kumimoto et al., 2008; Song et al., 2012a). ABA might affect how CO binds to the CORE elements and/or how CO recruits other factors, through different (but not necessarily incompatible) mechanisms.

ABA might generally regulate chromatin changes at the *FT* promoter. Increased levels of H3K4me3 and H3K9ac, representing active markers of gene expression (van Dijk et al.,

2010), and decrease nucleosome density occur in upregulated genes in response to drought stress. More specifically, both drought and ABA inducible genes, *RAB18*, *RESPONSIVE TO DESICCATION 20, 29A* and *29B* (*RD20, RD29A*, *RD29B*), show increased H3K4me3 and H3K9ac modifications under drought (Ding et al., 2012; Kim et al., 2012, 2008). Nucleosome density decrease has been described for *RD29A* and *RD20* (Kim et al., 2012). Also, H3K4me3 modifications are higher in the ABA biosynthetic gene *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) in response to drought (Ding et al., 2011). Direct links between ABA signalling and chromatin regulation are also emerging whereby SWI2/SNF2 chromatin remodelling ATPase BRAHMA (BRM) inactivation causes ABA hypersensitivity and ABA-related kinases SnRK2- dependent phosphorylation of BRM leads to its inhibition (Han et al., 2012). Interestingly, *brm* mutants display increased expression of *FT* (but also *CO*) suggesting a possible link between ABA and *FT* chromatin remodelling through regulation of BRM proteins (Farrona et al., 2004). In one possible model, ABA primes events at the *FT* chromatin (e.g. by modifying the chromatin status) to indirectly increase accessibility of CO to the CORE elements and promote *FT* transcriptional activation. The study of global and local changes in histone marks for active or inactive chromatin in the different ABA mutant backgrounds might help reveal whether ABA play any role in the remodelling of *FT* chromatin. However, as previously noted (Adrian et al., 2010), changes in chromatin marks may not provide causal indications about transcriptional regulatory events and rather be consequential to transcription factor binding activity at specific sites. In this respect, the use of CO-tagged transgenic lines in the different ABA backgrounds may be extremely informative to reveal if ABA facilitates the recruitment of CO to the CORE sites. This would be interesting in the light of recent reports describing how gibberellic acid, an important flowering hormone, regulates photoperiodic flowering through modulating the CO accessibility to the *FT*

promoter (Wang et al., 2016). This might point to a novel ABA-GA interplay in *FT* regulation.

It is important to consider that ABA alone is not sufficient to achieve high levels of *FT* activation, as our experiments indicate that CO requires GI for the ABA/drought-dependent activation of *FT* (Riboni et al., 2016, 2013). Thus, a more complex model (which is not necessarily incompatible with the one above) might be that ABA favours direct GI/CO protein interaction, thereby potentiating CO transcriptional activity through an unknown mechanism. This model might account for the reduced *FT* upregulation under drought stress of *4xcdf gi* mutants compared with *4xcdf* (Riboni et al., 2016). Furthermore, this mechanism might generally affect how CO and GI interact to activate gene expression. Several independent observations support this. First, lower levels of *FT* transcript were observed in *4xcdf gi* compared to *4xcdf* under normal watering conditions and such reduction cannot be fully explained in terms of diminished *CO* expression (Riboni et al., 2016). Secondly, the overexpression of *CO* cannot fully recuperate the late flowering of *gi* mutants nor *35S::CO* can confer high levels of *FT* expression in the absence of functional GI in LDs (Song et al., 2014a). Song and co-workers have shown that GI affects CO function by controlling its protein accumulation. Because I found no evidence of ABA controlling CO accumulation in Arabidopsis, and because my results indicate that ABA affects GI protein function (rather than its accumulation), I theorise that GI might have additional positive regulatory roles on CO action, and ABA might participate in these processes. A hypothetical model is represented in Fig. 16.

My confocal microscopy data indicate a role for CO in recruiting GI in nuclear speckles (Fig. 13D), in agreement with the reported physical interaction between CO and GI (Song et al., 2014a). CO is less efficient in re-localising FKF1 whereas GI promotes efficient re-localization of FKF1 from the nucleoplasm to GI-specific nuclear bodies, which are qualitatively and quantitatively different from those produced by CO (Fig. 13D-F).

Interestingly, in the presence of CO and GI, FKF1 becomes localised in the CO nuclear speckles (Fig. 13H). These data suggest sequential events for CO activation whereby CO acts as pull factor for the GI-FKF1 complex. Although my analysis cannot prove direct protein interactions, some evidences for the functional relevance of these putative protein complexes are beginning to emerge. GI directly binds to the *FT* promoter (Sawa and Kay, 2011) and interacts with CO *in vivo* (Song et al., 2014a). Similarly, FKF1 binds to the *FT* promoter near the CORE region (Song et al., 2012b) and also interacts with CO *in vivo* (Song et al., 2012b). If ABA had any role in GI-CO hetero-dimerization (Song et al., 2014a), recruitment of the FKF1-GI complex at positions occupied by CO might favour the proteasome degradation of repressor complexes like the CDFs at the *FT* promoter (Song et al., 2012b), thus boosting CO occupancy at the CORE elements. According to this model either lack of GI or reduced ABA would be limiting during *FT* activation.

Nuclear bodies in different eukaryotic systems are emerging as chromatin sites with active transcriptional activity (Kaiserli et al., 2015; Spector and Lamond, 2011). It is tempting to speculate that GI and ABA might cooperate in the regulation of CO (and perhaps CO-like proteins) to modulate gene expression. ABA might promote the activity of other proteins that bridge together CO and GI functions. For example, CO promotes *FT* transcription also through recruiting distal enhancer elements (Ben-Naim et al., 2006; Cao et al., 2014; Kumimoto et al., 2008; Song et al., 2012a). ABA-related transcription factors may facilitate favourable chromatin architecture for *FT* transcriptional activation. If demonstrated, it is also possible that such interplay between GI and ABA signalling on CO function can be extended to many other targets. Several deregulated genes in *co* mutants contain the CORE element, suggesting that the number of direct CO target genes is much higher than previously anticipated and that CO has role beyond photoperiodic flowering (Gnesutta et al., 2017; Wigge et al., 2005). Similarly, recent reports indicate that the GI-CDF is implicated in the regulation of different stress response genes (cold) (Fornara et al., 2015).

It is also worth noticing that GI also controls the ABA-dependent upregulation of the other florigen *TSF* but this does not seem to require CO function (Riboni et al., 2016). Thus, the postulated regulatory role of ABA on GI is not necessarily only connected to CO function and/or the photoperiodic pathway.

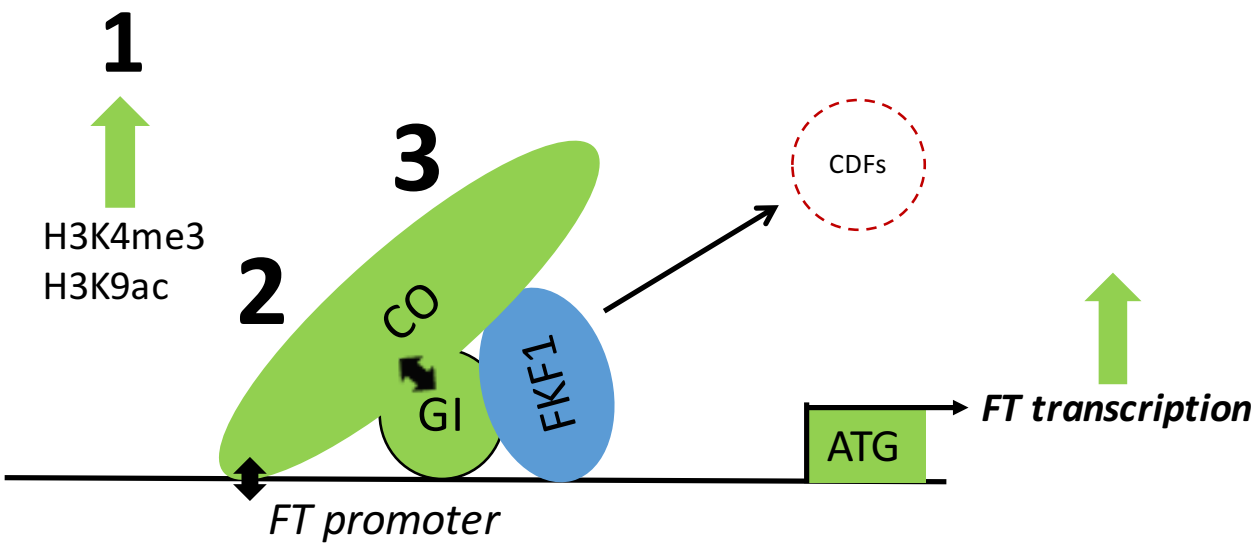

Fig. 16. Hypothetical mechanisms for the ABA-dependent activation of *FT*. Numbers represent three hypothetical roles of ABA on GI and CO functions. **1.** ABA might promote chromatin modifications, which facilitate CO accessibility to the *FT* promoter. **2.** ABA might stabilize the CO binding to the *FT* promoter through an unknown mechanism. **3.** ABA may enhance the CO-GI interaction, thus potentiating recruitment of FKF1 and reduction of repressive activity (e.g. by CDFs) at the *FT* promoter.

Multiple levels of ABA regulation of flowering through bZIPs transcription factors

The late flowering phenotype of *abf* plants (Fig. 15B) reveals that the canonical ABA signalling pathway is involved in regulating the floral transition. Interestingly, this result indicates that the ABFs are floral promoters confirming previous observation obtained with the *abf1 abf2 abf3 abf4* quadruple mutant (Yoshida et al., 2014). In that study, Yoshida and collaborators showed that *CO* expression is impaired in *abf1 abf2 abf3 abf4* quadruple mutants compared with the wild type both under drought stress and ABA treatment (Yoshida et al., 2014). To determine what proportion of the ABA positive role on flowering depends on the ABFs, I plan to analyse the effects of ABA deficit in triple mutants of *abf1 abf3 abf4* by generating *aba1 abf1 abf3 abf4* quadruple mutants. If ABA contributes to the floral network with both transcriptional and post-transcriptional effects on CO, I predict to observe an aggravation of the flowering phenotype of *aba1 abf1 abf3 abf4* quadruple mutants as compared with *abf1 abf3 abf4*.

Mutants in another ABA-related bZIP, *AREB3*, showed a mild flowering repressor activity (Fig. 15A and 15B) suggesting a more complex scenario in which different bZIPs affect the floral transition in opposite manners. However, the dramatic delay in flowering of *aba1-6 areb3* double mutants point to yet separate effects of *AREB3* in flowering, which are ABA-dependent (Fig. 15A). One could speculate that in the *aba1-6* background, lower levels of *FT* transcription highlight the role of *AREB3* as floral promoter which is usually masked by high *FT* transcript levels. Interestingly, *aba1-6 areb3* and *aba1-6 fd-4* double mutants show similar late flowering phenotypes compared to the wild type indicating that AREB3 might have FD-like function in the SAM (Fig. 15A). However, this molecular activity is only apparent under low ABA conditions. Another hypothesis is that AREB3 is required for florigen genes expression, although this does not account for the mild early flowering phenotype of *areb3* mutants. In this perspective, the analysis of the site of expression of

the different bZIPs and their mis-expression in plants will be crucial to distinguish between the two models.

3.4 Conclusions

My PhD work contribute to provide a molecular foundation for the interaction between ABA signalling and the photoperiodic pathway and generate the tools to address this at a deeper level. Although GI and CO are essential components of photoperiod flowering, the current view is that they form a transcriptional network of gene regulation. My data highlight the posttranscriptional control of this genetic pathway, and how this can be modulated by water inputs through ABA production. My published and unpublished data further point to a complex layer of flowering time regulation conferred by ABA, possibly in the shoot apical meristem. A positive role might be exerted through modulation of the FD-related bZIP factors. As a whole, my experiments highlight different roles of ABA in conveying water status information to modulate reproductive development.

3.5 Materials and Methods

Plant material and growing conditions

Arabidopsis thaliana plants used in this study are of ecotype Col-0. Mutant and transgenic lines were obtained from Nottingham Arabidopsis Stock Centre (NASC) or other laboratories or generated in this study as detailed in Table 1. Seeds were stratified and plants grown under long day conditions (LDs, 16 h light / 8 h dark), under controlled-environment cabinet, as previously described (Riboni et al., 2016). Temperature was set at 23 °C during the day and 19 °C in the night and air humidity was 60%. Two different growth chambers were used in this study, differing by light conditions. One chamber was fitted with fluorescent lamp (Philips, cool white) at an approximate fluency of 60 micro Einstein whereas in the other light was provided by a mix of cool white fluorescent tubes and Metal Halide high-intensity discharge 400W lamps (fluency was 250 micro Einstein). Because the penetrance of the *aba1* mutation in the flowering phenotype was reduced in the second chamber, I suspect an interaction might exist between light intensity and / or quality and ABA signaling in flowering time (which I could not investigate fully, due to inability to modifying light conditions in the same chamber).

ABA application experiments were performed following the procedure detailed previously (Riboni et al., 2016). Treatments were performed for 12 days and three different concentration of ABA were used: 0.25, 2.5 and 25 μM. A mock solution (0.025% v/v ethanol) afforded a negative control. For *in vitro Arabidopsis* growth Murashige and Skoog (MS) medium was prepared dissolving MS salt mix (Duchefa) and sucrose (1% w/v) in distilled water. The pH solution was adjusted to 5.8 and 0.8% w/v agar (Duchefa) was added. After autoclaving, the medium was mixed with Basta (50 μM final concentration) and poured into Petri plates. Sterilized seeds (70% v/v ethanol and 1% Sodium Dodecyl Sulphate, SDS for 10 minutes) were spread onto solidified agar plates, stratified for 2 days

(4°C and dark) and then moved to a growth chamber set as LDs. *Nicotiana benthamiana* plants used for transient expression experiments were grown under LDs in a greenhouse with semi-controlled climate.

Isolation of double mutants and genotyping

Double and triple mutants were generated by crossing. *aba1*-6, *phyA-501* and *phyB-9* mutations were genotyped as previously described (Martínez-García et al., 2014; Riboni et al., 2013; Strasser et al., 2009). The *cry2-1* allele was selected based on its late flowering phenotype and on the deletion present at the 5' of the gene (the primer couple indicated in Table 2 was generated on the deletion, so that annealing only occurs in the wild type allele, in absence of the deletion). *fd-4* and *fkf1-10* mutants were selected based on their late flowering phenotypes. *abf1*, *abf3*, *abf4* and *areb3* are T-DNA insertional mutations and the genotyping primers are listed in Table 2.

Molecular cloning

All full-length genes were cloned using the Gateway and Multi-Site Gateway (Three-fragment vector) cloning technology (Invitrogen) with primers listed in Table 2. The AttB1-AttB2 sites were added in two steps. A first PCR was performed using primers incorporating half of the nucleotide sequence complementary to the beginning/end of the gene of interest and the other half corresponding to half of the AttB1-AttB2 sites. PCR products were gel purified (Qiaquick Gel Extraction Kit, Qiagen) and used as templates for a second round of PCR with oligonucleotides with the complete AttB1-AttB2 sites. The Phusion High Fidelity DNA polymerase (New England Biolabs) was used for all the PCR reactions. Once the genes were cloned into the pDONR221 entry vectors (Invitrogen), an aliquot of the plasmid was sent for Sanger sequencing to ensure that no errors were

incorporated in the cloned sequence. The expression vector constructs generated in this study are listed in Table 3. The 5' and 3' elements and destination vectors were previously described; *SUC2 promoter / pDONR221 P4-P1r* (Marquès-Bueno et al., 2016), *2x35S / pDONR221 P4-P1r* and *mCITRINE / pDONR221 P2r-P3* (Jaillais et al., 2011), *2xmCherry-4xmyc / pDONR221 P2r-P3* (Simon et al., 2014), *pB7m34GW* and *pH7m34GW* (Karimi et al., 2017), *pGBPGWC* (Zhong et al., 2008), *pEarleyGate 102* (Earley et al., 2006). All the recombinant destination vectors were transformed into *Agrobacterium* cells, strain GV3101 (Koncz and Schell, 1986), for *Arabidopsis* transformation or *N. benthamiana* leaves infiltrations (see below). For CO heterologous production (see below Cell Free Degradation Assay), the full length *CO* (with stop codon) was expressed from an SP6 promoter and fused to N-terminus FLAG tag for expression with a transcription/translation TnT *in vitro* system compatible vector (the destination vector SP6::FLAG:GW was kindly provided by Kaiserli Lab (Kaiserli et al., 2015).

Plants transformation and BASTA selection

Destination vectors (Table 3), as well as controls *pGWB6* (Nakagawa et al., 2007) and *pSUC2::GW* (An et al., 2004), were introduced into *Agrobacterium* by electroporation. Transformed *Agrobacterium* were used to generate *Arabidopsis* transgenic plants via the floral dip technique (Clough and Bent, 1998). Transgenic plants were selected on the basis of Basta resistance conferred by the *bar* gene present in the destination vectors. Single insertion events were selected on Basta containing MS plates in which a Mendellian 3:1 ratio in T_2 generation was observed. T_3 homozygous lines were selected on MS+Basta plates, according to the absence of Basta resistance segregation.

Flowering time measurement

The number of rosette leaves produced by the plant was used to measure flowering time. To avoid flowering time alterations associated to MS-soil transfer, Basta resistance selection in T1 generation was performed directly on soil (and the same was done for informative T2 and T3 lines isolated from the analysis on MS plates with Basta). After germination, plants were sprayed with 100 μM Basta solution every other day until death of non-transgenic plants was observed.

Statistical analysis

Multiple comparisons among different genotypes were done with one-way ANOVA with Tukey's Post Hoc test, P-values ≤0.05 (*), ≤0.01 (**), ≤0.001 (***) to understand if differences in flowering time were statistically significant. Statistical analysis and plotting of the results were preformed using the GraphPad software.

Generation of isogenic lines

SUC2::HA:CO and *aba1-6 SUC2::HA:CO* isogenic lines were obtained by crossing a published *SUC2::HA:CO* line (Jang et al., 2009) with *aba1-6* mutants. In segregating F2 plants I selected homozygous *aba1-6* showing an early flowering phenotype (thus homozygous or heterozygous for the *SUC2::HA:CO* transgene). In the F3 generation, I screened lines that did not show segregation for the early flowering phenotype, and one was used for subsequent analysis. The *gi-2 GI::GI:HA* and *aba1-6 gi-2 GI::GI:HA* isogenic lines were generated by crossing the *gi-2 GI::GI:HA* line (Kim et al., 2007) with *aba1-6* mutants. In the F2 generation I selected *aba1-6* homozygous plants, which were not as late flowering time as *gi-2*. This flowering phenotype could correspond to different *GI* genotypes. Therefore, in the same generation, I selected *gi-2/gi-2* plants using primers

listed in Table 2. At this point individual *aba1-6 gi-2* double homozygous individuals could be either homozygous or heterozygous for the *GI::GI:HA* transgene. 15 F3 families were tested for the presence of the *GI::GI:HA* transgene in a homozygous state, based on lack of flowering time segregation. These corresponded to *aba1-6 gi-2 GI::GI:HA* triple homozygous and three independent F3 lines were in this way analysed in Figure 12.

RNA extraction, cDNA retrotranscription and real-time qPCR

Total RNA was extracted with QIAzol reagent (Invitrogen) and suspended in RNase-free milliQ dH_2O. RNA concentration was measured with a UV spectrophotometer and 500-750 ng aliquots were used for cDNA synthesis with the High Capacity RetroTranscriptase kit (Applied Biosystems). Quantitative real-time qPCR was performed as previously detailed (Riboni et al., 2013). Primers used to amplify *GI*, *CO*, *FT* and *TSF* were described in (Riboni et al., 2013), while those for *IPP2* and *ISU1* refer to (Kaiserli et al., 2015). *eGFP* and *RAB18* transcripts were amplified with the primers listed in Table 2.

Protein extraction and detection

50-80 mg of *gi-2 35S::HA:GI*, *gi-2 GI::GI:HA and aba1-6 gi-2 GI::GI:HA* seedlings grown on soil (under LDs) were harvested and immediately frozen in liquid nitrogen. Tissues were ground using glass beads with the TissueLyser II (Qiagen, 30 s at 28Hz shaking). 1 volume (100 μl per 100 mg) of Buffer E (Martínez-García et al., 1999) containing 0.1% (v/v) plant protease inhibitor cocktail (SIGMA) was added to each sample and mixed. After 10 min of centrifugation at maximum speed (*18,000 x g*), the supernatant was recovered and an aliquot was used to measure the concentration of the protein extracts with the Bradford reagent. Bovine Serum Albumin (BSA) at known concentrations were used as a standard reference. Proteins were mixed with Laemmli Buffer 4X (Invitrogen) and

incubated for 10 minutes at 75°C. The same amount of proteins was loaded onto a 7% SDS-PAGE gel. For Western blot analysis, monoclonal Anti-HA antibody (SIGMA) was used to detect HA:GI signal. Ponceau red staining of Rubisco large subunit or Anti-H3 antibody (Agrisera) for Histone H3 signal detection were used as loading controls. Chemiluminescent signals were detected through ChemiDoc Touch Imaging System (BIORAD).

Nuclei isolation and CO protein detection in *Arabidopsis*

Several attempts were made to detect CO:CFP and HA:CO protein in *Arabidopsis* transgenic plants. Total protein extraction with the Buffer E protocol, previously described for *GI* and optimal to detect CO:CFP when transiently expressed *N. benthamiana* leaves, did not work in *Arabidopsis*. I also tried different nuclei isolation protocols described in the literature that yielded unsatisfactory results with and non-specific signals. After different trials I realized that the isotype of Anti-GFP or Anti-HA antibody was crucial to obtain results, and I finally set up a working protocol to detect CO:CFP and HA:CO in my transgenic plants (although in general CO:CFP plants yielded much reduced signal). According to my optimized procedure, approx. 100 mg of *Arabidopsis* seedlings were harvested and immediately frozen in liquid nitrogen. Tissue samples were ground by shaking with glass beads in a TissueLyser II (Qiagen, 2 pulses of 30 s each at 28Hz shaking). Leaf powder was suspended in 1.2 ml of cold nuclear isolation buffer (20 mM Tris-HCl, pH = 8.8, 25 mM NaCl, 5 mM $MgCl_2$, 30% (v/v) glycerol, 5% (w/v) sucrose, 0.5% (v/v) Triton X-100, 0.08% (v/v) β-mercaptoethanol, 0.2% (v/v) SIGMA plant protease inhibitor, 1mM DTT, 1.3 mM PMSF). The samples were filtrated trough two layers of Miracloth (Millipore) and centrifuged at *5,000 x g*, at 4 °C, for 10 minutes. The supernatant was removed and the pellet was washed four times with 1 ml of nuclear isolation buffer

and, after each wash, pelleted at 4 °C at decreasing speed: *5,000 x g*, *2,700 x g*, *2,200 x g* and *2,200 x g*, 8 minutes each time. Nuclei were suspended in 30 μl of nuclear isolation buffer, mixed with 10 μl of Laemmli Buffer and heated 10 minutes at 95 °C. Samples were centrifuged for 1 minute at *3,000 x g* to pellet all nuclear membranes, and the supernatants (enriched in soluble nuclear proteins) were recovered and 20 μl were loaded onto a 10% SDS-PAGE gel. For Western blot analysis, anti-HA-Peroxidase, High Affinity antibody (Roche) was used to detect HA:CO protein signal and anti-GFP antibody (Abcam ab6556) was used to detect the CO:CFP protein signal. Chemiluminescent signals were detected through a ChemiDoc Touch Imaging System (BIORAD) and measured by Image Lab software.

Transient expression in *N. benthamiana*

4-5 week-old *N. benthamiana* leaves were infiltrated with different combinations of transformed *Agrobacterium* as described in (Sparkes et al., 2006). 10mM $MgCl_2$ solution was used as infiltration medium and *Agrobacterium* transformed with P19 plasmid was added to the final infiltration mixture to suppress gene silencing. Infiltrated *N. benthamiana* plants were grown under LDs for three more days before proceeding with ABA treatment experiments or confocal microscopy analysis.

ABA treatment and CO:CFP protein detection in *N. benthamiana*

Agrobacterium carrying the *35S::CO:CFP* or *35S::GFP:GW* expression constructs were infiltrated in *N. benthamiana* leaves as previously described. At least 6 independently infiltrated leaves were piled up together and leaf punches of 5 mm in diameter were collected on a petri dish containing liquid MS medium supplied with 0.01% (v/v) Silwet L-77 detergent. Leaf disks were then randomly divided in petri dishes containing liquid MS (+

0.01% (v/v) Silwet L-77 detergent) supplemented with different concentrations of phyto-hormones (ABA, GA_4 or JA) or an appropriate diluent (mock = 0.025% (v/v) ethanol). Depending on the experiment, at each time point 12-20 leaf disks were harvested and immediately frozen in liquid nitrogen. Frozen tissues were ground using glass beads in the TissueLyser II bead beater (Qiagen, 2 pulses of 30 s each at 30Hz shaking) and total proteins were extracted with the buffer E protocol as previously described. Equal amounts of proteins were loaded onto a 10% SDS-PAGE gel. For Western blot analysis, anti-GFP antibody (Abcam ab6556) was used to detect CO:CFP or GFP protein signal. Ponceau red staining of Rubisco large subunit or Anti-UGPase antibody (Agrisera) for UGPase signal detection were used as loading controls. Chemiluminescent signals were captured on western blot compatible films.

Confocal microscopy

Fluorescence signals from *N. benthamiana* infiltrated leaves were imaged with a Leica FRET-FLIM confocal microscope. CFP (Cerulean) was exited at 405 nm and its emission was collected between 405 and 550 nm; mCITRINE was exited at 488 nm and its emission was collected between 488 and 520 nm; mCherry was exited at 552 nm and its emission was collected between 600 and 780 nm. To avoid the overlap between CFP and CITRINE fluorescence signals, when CO:CITRINE, FKF:CFP and GI:CHERRY were co-expressed in the same cell CFP was exited at 405 nm and its emission was collected between 405 and 500 nm, while CITRINE was exited at 488 nm and its emission was collected between 510 and 530 nm; All the confocal microscopy work was performed at Kaiserli Lab at the University of Glasgow during my Erasmus+ project. *Agrobacterium*-containing infiltration mixtures were prepared combining differently transformed *Agrobacterium* according to the co-localization I wanted to monitor. The expression constructs used are listed in Table 3. *35S::TZP:cherry* construct was kindly provided by Kaiserli Lab. For all the combinations

tested, three independent transient expression experiments were performed in which I monitored the fluorescence signals in 4/5 different cells.

Cell Free Degradation Assay

1g of 10 day-old wild type *Arabidopsis* seedlings grown on non-selective MS solid medium under LDs were harvested and immediately frozen in liquid nitrogen. Frozen tissues were ground in liquid nitrogen with mortar and pestle and total proteins were extracted in degradation buffer as described in (Wang et al., 2009). Equal amounts of heterologous FLAG:CO produced with the TNT SP6 High-Yield Wheat Germ protein expression system was mixed with 200 μg of soluble plants proteins (around 100 μl) supplemented with proteasome inhibitor (50 μM MG132) or 0.1% (v/v) SIGMA plant protease inhibitor (PIC). The degradation reactions were incubated at 22 °C and samples collected after 5-15-30-60-120 minutes. 20 μl of the reaction was mixed with Laemmli Buffer 4X and heated at 75 °C for 10 minutes. An aliquot of total protein extract before and immediately after the addiction of FLAG:CO was used as negative control and starting condition (T0), respectively. Equal volumes were loaded onto a 10% SDS-PAGE gel. For Western blot analysis, anti-FLAG antibody (Abcam ab49763) was used to detect FLAG:CO protein signal through a ChemiDoc Touch Imaging System (BIORAD). Chemiluminescent signals were measured by Image Lab software.

Table 1

Allele	Reference
aba1-6	(Niyogi et al., 1998)
aba2-1	(Léon-Kloosterziel et al., 1996)
phyA-501	(Ruckle et al., 2008)
aba1-6 phyA-501	This work
phyB-9	(Reed et al., 1993)
aba1-6 phyB-9	This work
fkf1-10	(Riboni et al., 2013)
aba1-6 fkf1-10	This work
cry2-1	(Guo et al., 1999)
aba1-6 cry2-1	This work
hab1-1 abi1-2 abi2-2	(Rubio et al., 2009)
areb3	SALK_061079C (NASC)
aba1-6 areb3	This work
abf1	SALK_132819C (NASC)
abf3	SALK_096965 (NASC)
abf4	SALK_069523 (NASC)
abf1 abf3 abf4	This work
fd-4	(Riboni et al., 2013)
aba1-6 fd-4	This work
Col-0 35S::GW:CFP	This work
35S::CO:CFP	This work
aba1-6 35S::CO:CFP	This work
hab1-1 abi1-2 abi2-2 35S::CO:CFP	This work
Col-0 SUC2::GW	This work
aba1-6 SUC2::GW	This work
aba2-1 SUC2::GW	This work
SUC2::CO:CITRINE	This work
aba1-6 SUC2::CO:CITRINE	This work
aba2-1 SUC2::CO:CITRINE	This work
SUC2::HA:CO	(Jang et al., 2009)
aba1-6 SUC2::HA:CO	This work
gi-2 GI::GI:HA	(Kim et al., 2007)
aba1-6 gi-2 GI::GI:HA	This work
gi-2 35S:HA:GI	(David et al., 2006)

Table 2

Target	Forward primer	Reverse primer	Application
cry2-1	CTGGAGGAGGTTGAGGTCTG	CCAAGAGCCTTCAAGGATTG	Genotyping
GI	GTACAGCAAGGAAGCTCATCC	CAGTTTTATAAATGGGACGGTT	Genotyping
gi-2	CGCATTTTGACTCATTACAATT	CATAGACCTCAGCAGAGAGACC	Genotyping
ABF1	AGAGGGAATGAGTCAAAGCC	TTGCCTTCTCTCAACAACCT	Genotyping
abf1	AGAGGGAATGAGTCAAAGCC	TGGTTCACGTAGTGGGCCATCG	Genotyping
ABF3	TTGCCTCGGACGATTAGTCA	GGAACAGGGGACAAAGATGC	Genotyping
abf3	TGGTTCACGTAGTGGGCCATCG	GGAACAGGGGACAAAGATGC	Genotyping
ABF4	CGCACGCATTTATGTGGTTTG	GTTTCCGTTGACCTGACCCA	Genotyping
abf4	CGCACGCATTTATGTGGTTTG	TGGTTCACGTAGTGGGCCATCG	Genotyping
AREB3	TGATGAGCAGGCTTACACTC	GCCTACAAGAAGAAGGCTTTGC	Genotyping
areb3	TGGTTCACGTAGTGGGCCATCG	GCCTACAAGAAGAAGGCTTTGC	Genotyping
eGFP	ACGTAAACGGCCACAAGTTC	AAGTCGTGCTGCTTCATGTG	qPCR
RAB18	TCGGTCGTTGTATTGTGCTTTTT	CCAGATGCTCATTACACACTCATG	qPCR
CO (with STOP)	AAAAAGCAGGCTTCACCATGTTGAAACAAGAGAGTAAC	AGAAAGCTGGGTTTCAGAATGAAGGAACAATCC	Cloning
CO	AAAAAGCAGGCTTCACCATGTTGAAACAAGAGAGTAAC	AGAAAGCTGGGTTGAATGAAGGAACAATCC	Cloning
FKF1	AAAAAGCAGGCTTCACCATGGCGAGAGAACATGCGATC	AGAAAGCTGGGTTCAGATCCGAGTCTTGCCGG	Cloning
GI	AAAAAGCAGGCTTCACCATGGCTAGTTCATCTTCATCTGAG	AGAAAGCTGGGTTTTGGGACAAGGATATAGTACAGC	Cloning
Adapter AttB1/AttB2	GGGGACAAGTTTGTACAAAAAAGCAGGCT	GGGGACCACTTTGTACAAGAAAGCTGGGT	Cloning

Table 3

Expression construct	Entry clone 1 (5' element)	Entry clone 2	Entry clone 3 (3' element)	Destination vector	Applications
35S::CO:CFP		*CO / pDONR207 P1-P2*		pGBPGWC	*Arabidopsis* transformation & *N. benthamiana* transient expression
SUC2::CO:CITRINE	*SUC2 promoter / pDONR221 P4-P1r*	*CO / pDONR207 P1-P2*	*mCITRINE / pDONR221 P2r-P3*	pB7m34GW	*Arabidopsis* transformation
35S::CO:CITRINE	*2x35S / pDONR221 P4-P1r*	*CO / pDONR207 P1-P2*	*mCITRINE / pDONR221 P2r-P3*	pH7m34GW	*N. benthamiana* transient expression
35S::CO:cherry	*2x35S / pDONR221 P4-P1r*	*CO / pDONR207 P1-P2*	*2xmCherry-4xmyc / pDONR221 P2r-P3*	pH7m34GW	*N. benthamiana* transient expression
35S::GI:cherry	*2x35S / pDONR221 P4-P1r*	*GI / pDONR221 P1-P2*	*2xmCherry-4xmyc / pDONR221 P2r-P3*	pH7m34GW	*N. benthamiana* transient expression
35S::FKF1:cherry	*2x35S / pDONR221 P4-P1r*	*FKF1 / pDONR207 P1-P2*	*2xmCherry-4xmyc / pDONR221 P2r-P3*	pH7m34GW	*N. benthamiana* transient expression
35S::FKF1:CFP		*FKF1 / pDONR207 P1-P2*		pEarleyGate 102	*N. benthamiana* transient expression

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Part II

Published Paper I

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Environmental stress and flowering time: the photoperiodic connection. Modulation of florigen expression upon environmental stress

Matteo Riboni[1], Alice Robustelli Test[1], Massimo Galbiati[1,2], Chiara Tonelli[1], Lucio Conti[1]

1 = Department of Biosciences
Università degli Studi di Milano
Via Celoria, 26
20133, Milan, Italy

2 = Fondazione Filarete,
Viale Ortles 22/4
20139, Milan, Italy

MINI-REVIEW

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Environmental stress and flowering time
The photoperiodic connection

Matteo Riboni[1], Alice Robustelli Test[1], Massimo Galbiati[1,2], Chiara Tonelli[1], and Lucio Conti[1,*]

[1]Department of Biosciences; Università degli Studi di Milano; Milan, Italy; [2]Fondazione Filarete; Milan, Italy

Keywords: plant stress response, Florigen, drought escape, plant adaptive development, photoperiodic flowering

Plants maximize their chances to survive adversities by reprogramming their development according to environmental conditions. Adaptive variations in the timing to flowering reflect the need for plants to set seeds under the most favorable conditions. A complex network of genetic pathways allows plants to detect and integrate external (e.g., photoperiod and temperature) and/or internal (e.g., age) information to initiate the floral transition. Furthermore different types of environmental stresses play an important role in the floral transition. The emerging picture is that stress conditions often affect flowering through modulation of the photoperiodic pathway. In this review we will discuss different modes of cross talk between stress signaling and photoperiodic flowering, highlighting the central role of the florigen genes in this process.

Photoperiodic-Dependent Activation of Flowering

After the floral transition the shoot apical meristem (SAM) changes its identity switching from vegetative to reproductive. In annual *Arabidopsis* ecotypes, the transition to flowering is strongly promoted by variations in day length (photoperiod). The photoperiodic pathway promotes flowering when *Arabidopsis* plants are exposed to long days (LDs) conditions (typical of spring and summer). Photoperiodic flowering is the result of complex interactions between the circadian clock (an endogenous timekeeping mechanism) and external cues, which ultimately results in the activation of a set of floral genes.[1] Central to photoperiod-dependent flowering is the pattern of accumulation of the flowering protein CONSTANS (CO).[2-4] *CO* expression is regulated transcriptionally by the circadian clock through the GIGANTEA (GI)-FLAVIN-BINDING, KELCH REPEAT, F-BOX (FKF1) complex.[5,6] LDs also promote the stabilization of CO protein at the end of a LD via activation of the photoreceptors PHYTOCROME A, CRYPTOCHROME 1 and 2 (CRY1 and 2).[3] CO protein promotes the transcriptional activation of the florigen genes *FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*) in the phloem companion cells.[7-10] FT and FT-likes proteins encode small proteins with similarity to the Raf Kinase Inhibitor Proteins (RKIP). They usually act as systemic signals, since these proteins are able to move between cells.[11] FT protein moves from the leaves to the SAM where it interacts with the SAM-specific bZIP transcription factors FLOWERING LOCUS D (FD) and FD PARALOG (FDP) to initiate the floral transition.[12-16] Here, the FT/FD heterodimer activates several MADS box-type transcription factors, namely *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA1*, and *FRUITFUL*, responsible for triggering the floral transition.[17,18]

Florigen gene expression has been demonstrated to play a pivotal role in photoperiodic flowering in different plants including *Arabidopsis*, a facultative LD plant and Rice (*Oryza sativa*), a facultative short day (SD) plant.[19] However, florigen expression is not always dependent upon photoperiod variations as in the case of the day neutral plant Tomato (*Solanum lycopersicum*).[20] This implies that florigen upregulation can also occur in response to internal or external stimuli other than variations in day length. The data reviewed here reinforces the idea that the photoperiodic pathway and the florigen genes are central nodes of a wider network receiving a multitude of external inputs. Furthermore, mechanisms that couple photoperiodic flowering with stress acclimation are emerging.

Stress-Dependent Activation of *FT* Expression

LDs promote flowering via activation of the florigen genes in *Arabidopsis*. However, it is now apparent that the *FT* promoter conveys several environmental information, in some cases independent of day length. Many plant species are induced to flower following drought stress which results in a drought escape response - DE -.[21-27] The onset of DE maximizes the chances to set seeds, thus "escaping" from a potentially lethal drought condition.[28] We have recently shown that in *Arabidopsis* DE occurs under LDs but not SDs, thus revealing a strong interdependence of certain drought responses on photoperiod. Genetic screens showed that photoperiod-stimulated GI activity is necessary and sufficient to trigger a drought dependent activation of the florigen genes *FT* and *TSF*.[29]

The phytohormone ABA plays a pivotal role in mediating several drought adaptive mechanisms although its precise role in flowering is still poorly understood.[30] Genetic and expression

*Correspondence to: Lucio Conti; Email: Lucio.Conti@unimi.it
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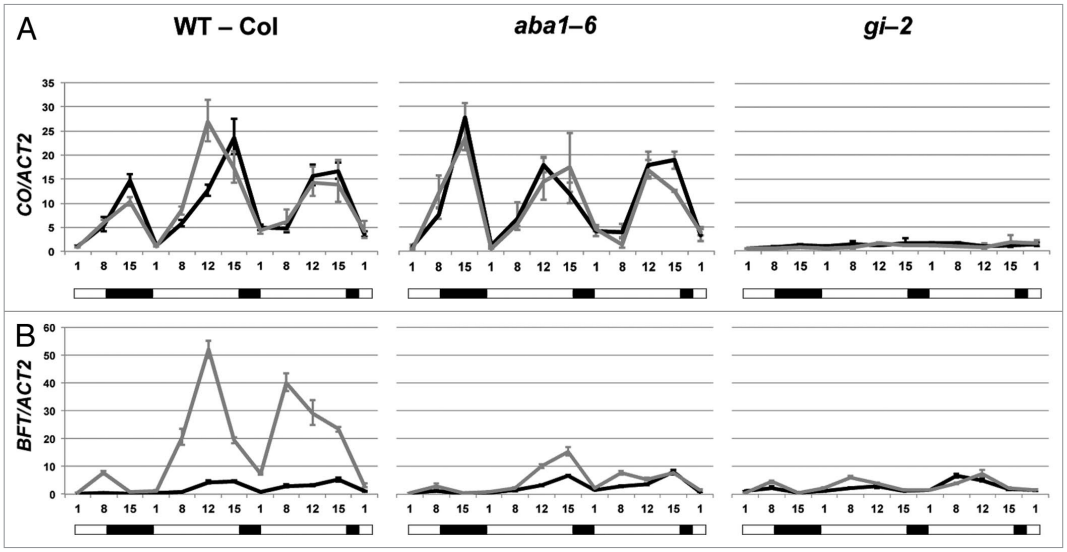

Figure 1. Real-time qPCR of *CO* (**A**) or *BFT* (**B**) transcripts in 3 wk-old wild-type (Col-0), *aba1-6* or *gi-2* seedlings. Plants were subjected to normal watering (black lines) or reduced watering (gray lines) regimes and harvested at the indicated time points in coincidence with the light phase (open bar) or in the dark (black bar) during a SDs to LDs shift. At each time point, values represent fold change variations of *CO* or *BFT* transcript levels relatively to Col-0 under NW. *ACT2* expression was used for normalization; error bars represent SD of 2 technical replicates. A representative experiment of 2 biological replicates is shown.

data suggest a role for ABA in DE response, through the activation of the florigen genes.[29] *aba1* mutants are impaired in ABA biosynthesis and display reduced accumulations of *FT* and *TSF* transcripts, especially under drought conditions. In addition to *FT* and *TSF* another *FT*-like genes *MOTHER OF FT AND TFL1* (*MFT*) all appear to be positively regulated by ABA.[31,32] Taken together these data argue in favor for a positive role for endogenous ABA in flowering via potentiation of florigen-like genes in a photoperiodic manner.

Some plants use drought stress as a primary cue to flowering. Recent studies suggest that drought stress is involved in the upregulation of the florigen genes in the tropical tree *Shorea beccariana*.[33] Moderate increases in drought index promote an increase of *SbFT* transcript accumulations early in bud development, preceding flower morphological changes. *Shorea beccariana* grows at the equator where day length and temperature are constant throughout the year. It is thus plausible that drought spells could represent a major external cue to trigger mass flowering in this species via direct activation of *FT* independent of photoperiod. Photoperiod-independent modes of activation of *FT* exist also in *Arabidopsis* where an increase in ambient temperature is reflected in augmented *FT* transcript accumulation.[34] A key component of this mechanism is the bHLH transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) directly activating *FT* expression largely independent of CO.[35] It is intriguing to note that occurrence of drought episodes often coincides with an increase in ambient temperature, at least in temperate climates. Whether ambient temperature also plays a regulatory role in DE response is thus an interesting question.

Unlike the thermosensory pathway, the mechanism through which drought stimuli affect *FT* activation is unknown. Drought stress results in an increase in *FT* expression with no evident effect on the physiological circadian oscillation of *FT*.[29,36] Because the pattern of *FT* transcript accumulation depends on variations in CO protein, drought might directly affect *CO* expression. FLOWERING BHLH 1 (FBH1), a *CO* positive activator, is phosphorylated in vivo following ABA signaling activation.[37,38] Although the precise role of phosphorylation on FBH1 protein function is still unknown, this finding could support a role for ABA in *CO* transcription under drought conditions. Also, EID1-like protein 3 (EDL3), a positive regulator of ABA signaling is an activator of *CO*. *EDL3* transcript is upregulated following ABA applications.[39] Although these findings point to a link between ABA and photoperiodic flowering via *CO* transcript accumulations we could find only minor variations in *CO* transcript in wild-type or *aba1* mutant plants subjected to drought stress (**Fig. 1A**).

Drought (via ABA) could affect CO protein activity or stability. For example, besides the well-established role in seed germination the ABA signaling protein ABA INSENSITIVE 3 (ABI3) is involved in the control of flowering time. *abi3* mutants are early flowering under both SDs and LDs while *ABI3* overexpression results in an increased vegetative phase under LDs.[40] ABI3 binds to the CO CCT (CO, CO-like, TOC1) domain involved in the recruitment of the CO protein to the promoter

of *FT*.[41,42] Thus, interaction with ABI3 may interfere with CO (and perhaps other CCT domain - containing proteins) binding to DNA. Intriguingly, following spray with ABA, *abi3* mutants display high levels of *TSF*, suggesting a repressive role for ABI3 on *TSF* expression.[43] In germinating seeds, the expression of *MFT* is downregulated by ABI3.[31] ABI3 may thus act as a negative regulator of flowering through downregulation of florigen-like genes.

Despite the GI-CO module being responsible for most of the activation of *FT*, *FT* upregulation may occur independently of either CO or GI. For example, warm temperatures results in an acceleration of flowering in the absence of GI and CO activities.[34] In contrast, a DE response can be induced in *co* but not *gi* mutants, although it is unknown whether drought can stimulate *FT* upregulation in the absence of CO activity.[29] Nonetheless this observation suggests that drought signals can overcome CO action to trigger flowering, provided that GI is photoperiod-stimulated. In support of the key role of GI in DE, ABA hypersensitive mutants are early flowering under LDs, but not under SDs. Thus ABA hyper-activation cannot override the requirement of photoperiod-stimulated GI in flowering.[29] Examples of GI dependent but CO-independent mechanisms for *FT* activation have been described.[35,44-48] However it is currently unclear how drought might affect GI-derived signals upon the *FT* promoter. Other pathways could facilitate the responsiveness of *FT* to photoperiod-stimulated GI. For example, similarly to *gi*, *cry2* mutants have a defective DE response, despite constitutively accumulating increased ABA levels compared with wild type.[29,49] Therefore, one could speculate that also CRY2 may participate in the GI- and ABA-dependent activation of *FT*.

Arabidopsis has 3 florigen genes, of which 2 (*FT* and *TSF*) act redundantly to mediate photoperiodic flowering.[8,50,51] Despite this functional redundancy, *FT* and *TSF* transcripts are found in a non-overlapping pattern of expression.[8] Also, *TSF* expression (but not *FT*) can be activated under SDs following exogenous applications of a synthetic Cytokinin (CK).[52] Thus, unlike ABA, CKs do not require a photoperiodic input for the activation of *TSF*. Because of this reduced dependence on photoperiod, *TSF* upregulation might also occur in the absence of CO (although still in a GI-dependent manner) under drought conditions and contribute to the DE response observed in *co* mutants. In conclusion, more work is needed to clarify the mode of *FT* and *FT-like* genes activations under drought conditions and their specific interdependence with the photoperiodic pathway machinery.

Stress Dependent Downregulation of *FT* Expression

Not all abiotic stresses are interpreted as an escape signal. For example, cold stress delays flowering and alters the diurnal oscillation of *FT* expression even under inductive photoperiodic conditions. It has been shown that cold temperatures induce the degradation of CO protein via an ubiquitin/proteasome pathway that involves the E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1).[53] Under normal growth temperature HOS1 acts as a general component of photoperiodic flowering by destabilizing CO protein in response to daylight signals.[54] Modulation of HOS1 activity by light and cold temperature plays a crucial role in the daily pattern of CO accumulation, thus revealing yet another example of interplay between environmental cues and day length perception via florigen regulation.

A different osmotic stress, salinity, delays flowering in *Arabidopsis* by interfering with the photoperiodic pathway. Interestingly salt affects *FT* at 2 different levels, transcriptional and post-transcriptional. Salt stress promotes GI protein degradation through an unknown ubiquitin/proteasome pathway.[55] Consequently, salt negatively regulates *CO* and *FT* transcripts accumulation. Salt stress delays flowering by activating the floral repressor *BROTHER OF FT* (*BFT*), a florigen-like protein with opposite function to *FT*.[56] BFT competes with FT for the binding to FD, thus delaying the switch to flowering. *BFT* is strongly responsive to drought stress and ABA.[57] We also confirmed that *BFT* can be transcriptionally activated under drought conditions in an ABA dependent manner and this regulation is dependent on GI (**Fig. 1B**). Thus, *BFT* expression is subject to a similar regulatory mechanism that orchestrates the activation of *FT* and *TSF* and is responsible for the DE response. However, the physiological role of *BFT* in DE is unclear since under drought conditions the positive regulation of flowering (i.e., via *FT* and *TSF*) clearly prevails over *BFT*. One could hypothesize that the balance between florigen and anti-florigen proteins is necessary to generate an optimal duration of reproductive development according to environmental stress. In this sense BFT may buffer FT action and prevent a premature interruption of inflorescence development. Deciphering the regulatory logic of the different florigen genes is thus an important goal to gain insights into the different flowering adaptations to stress as well as the mechanisms that govern crop seed yield under adverse conditions.

Future Challenges: Coordination of Escape and Tolerance Strategies

A question arise as to how plants might coordinate flowering networks with tolerance responses, which allow individual cells to survive under stress conditions. GI is emerging as a key node connecting different abiotic responses with flowering time. *gi* mutants display different phenotypes including an increased salt tolerance.[55] GI directly binds to SALT OVERLY SENSITIVE 2 (SOS2) protein and prevents its action under normal growth condition. Salt stress triggers the degradation of GI, thus releasing SOS2 and activating a salt-stress tolerance pathway. Besides salt, GI affects several developmental transitions (e.g., seedling photomorphogenesis and flowering time) as well as different environmental responses (starch accumulation, sucrose metabolism, sensitivity to light and oxidative stress).[48,58-62] Furthermore GI controls guard cell activity.[63] GI could coordinate different responses through a process of

sequestration and release of interacting partners.[55] In this model GI stability plays a key role through which plants can coordinately regulate independent processes with flowering.

In conclusion, plant adaptation to stress is complex and involves different strategies. In *Arabidopsis* the escape strategy requires a positive integration between photoperiodic and drought-dependent signals. A floral delay strategy takes place upon conditions where growth restraint provides an adaptive advantage over an escape, namely on salt.[64] In all these cases, modulation of florigen genes represents the common central thread for how differential flowering strategies are enacted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Matteo Riboni*, Alice Robustelli Test, Massimo Galbiati, Chiara Tonelli, and Lucio Conti†

Department of BioSciences, Università degli Studi di Milano, Via Celoria 26, 20133, Milan, Italy

* Present address: Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, South Australia 5064, Australia.

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RESEARCH PAPER

ABA-dependent control of *GIGANTEA* signalling enables drought escape via up-regulation of *FLOWERING LOCUS T* in *Arabidopsis thaliana*

Matteo Riboni*, Alice Robustelli Test, Massimo Galbiati, Chiara Tonelli, and Lucio Conti†

Department of BioSciences, Università degli Studi di Milano, Via Celoria 26, 20133, Milan, Italy

* Present address: Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, South Australia 5064, Australia.
† Correspondence: lucio.conti@unimi.it

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Abstract

One strategy deployed by plants to endure water scarcity is to accelerate the transition to flowering adaptively via the drought escape (DE) response. In *Arabidopsis thaliana*, activation of the DE response requires the photoperiodic response gene *GIGANTEA* (*GI*) and the florigen genes *FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*). The phytohormone abscisic acid (ABA) is also required for the DE response, by promoting the transcriptional up-regulation of the florigen genes. The mode of interaction between ABA and the photoperiodic genes remains obscure. In this work we use a genetic approach to demonstrate that ABA modulates GI signalling and consequently its ability to activate the florigen genes. We also reveal that the ABA-dependent activation of *FT*, but not *TSF*, requires *CONSTANS* (*CO*) and that impairing ABA signalling dramatically reduces the expression of florigen genes with little effect on the *CO* transcript profile. ABA signalling thus has an impact on the core genes of photoperiodic signalling *GI* and *CO* by modulating their downstream function and/or activities rather than their transcript accumulation. In addition, we show that as well as promoting flowering, ABA simultaneously represses flowering, independent of the florigen genes. Genetic analysis indicates that the target of the repressive function of ABA is the flowering-promoting gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), a transcription factor integrating floral cues in the shoot meristem. Our study suggests that variations in ABA signalling provide different developmental information that allows plants to co-ordinate the onset of the reproductive phase according to the available water resources.

Key words: Abscisic acid (ABA), adaptation, drought stress, florigen expression, flowering, photoperiod.

Introduction

Water deprivation triggers several physiological adjustments at the cellular and organ levels (Shinozaki and Yamaguchi-Shinozaki, 2007). Depending on the intensity and duration of drought episodes, some plants can also respond adaptively, by activating the drought escape (DE) response (Franks, 2011; Riboni *et al.*, 2013, 2014; Kazan and Lyons, 2016). DE allows plants to accelerate the floral transition and set seeds before drought conditions become too severe. While escaping

Abbreviations: DE, drought escape; LD, long day;SD, short day.

the potentially lethal effects of drought, plants undergoing DE usually produce fewer fruits and seeds, indicating a trade-off between plant survival and successful seed set (Su *et al.*, 2013; Kenney *et al.*, 2014). Therefore, a more precise understanding of the mechanisms leading to DE is of fundamental importance to assess the diverse modes of adaptations of natural plant populations as well as to produce crops with increased productivity under water deprivation (Lovell *et al.*, 2013; Kooyers, 2015).

Arabidopsis thaliana is a facultative long-day (LD) plant, flowering much earlier under LDs, typical of spring/summer compared with short days (SDs). The DE response occurs under LDs, but not SDs, indicating an interdependence between DE and photoperiod signalling in Arabidopsis (Han *et al.*, 2013; Riboni *et al.*, 2013). The photoperiodic pathway comprises three key genes, whose regulation and activity are required for the correct interpretation of day length: *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). *CO* encodes a nuclear protein (Putterill *et al.*, 1995; Samach *et al.*, 2000) able to induce the transcriptional activation of the florigen genes *FT* and *TWIN SISTER OF FT* (*TSF*) (An *et al.*, 2004; Yamaguchi *et al.*, 2005; Jang *et al.*, 2009). Accumulation of the *CO* transcript during the day depends on LIGHT OXYGEN VOLTAGE (LOV) domain-containing, blue light receptor FLAVIN-BINDING, KELCH REPEAT F-BOX 1 (FKF1), and GI (Imaizumi *et al.*, 2003, 2005; Sawa *et al.*, 2007; Fornara *et al.*, 2009; Song *et al.*, 2012). Formation of a GI–FKF1 complex is stimulated by blue light and leads to degradation of the *CO* transcriptional repressors CYCLING DOF FACTORs (CDFs) (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009), allowing *CO* transcription. While *CO* transcript accumulation broadly occurs under both LDs and SDs, CO protein is activated to promote flowering only under LDs when *CO* mRNA peaks in the light phase at the end of the day (Suarez-Lopez *et al.*, 2001). Such a daily pattern of CO protein accumulation is controlled by several types of photoreceptors, which generate a peak of CO abundance in coincidence with dusk under LDs (Valverde *et al.*, 2004; Jang *et al.*, 2008; Liu *et al.*, 2008; Zuo *et al.*, 2011; Lazaro *et al.*, 2012; Song *et al.*, 2012).

CO promotes *FT* transcription in the phloem companion cells (Adrian *et al.*, 2010). However, FT protein acts as a florigenic signal, moving long distance to the shoot apical meristem (SAM), where it interacts with the bZIP transcription factors FLOWERING LOCUS D (FD) and FD PARALOGUE (FDP) 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; Jaeger *et al.*, 2013). Amongst the early targets of the FT–FD complex is *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), a MADS box transcription factor, which integrates several floral pathways in the SAM (Borner *et al.*, 2000; Lee *et al.*, 2000; Samach *et al.*, 2000; Moon *et al.*, 2003; Searle *et al.*, 2006; Jang *et al.*, 2009; Wang *et al.*, 2009; Lee and Lee, 2010).

Besides photoperiod, *FT* activation is modulated by several environmental cues (Pin and Nilsson, 2012), including drought stress (Riboni *et al.*, 2013). The activation of *FT* by drought requires abscisic acid (ABA), a key hormone mediating water stress stimuli (Riboni *et al.*, 2013). ABA derives from the carotenoid zeaxanthin synthetized in chloroplasts. Here, different enzymes, including ABA1, transform zeaxanthin into xanthoxin prior to its translocation to the cytoplasm where another set of enzymes, namely ABA2, complete the last biosynthetic steps leading to bioactive ABA (Nambara and Marion-Poll, 2005). Three signalling proteins form the core ABA signalling, including the PYRABACTIN RESISTANCE (PYR)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR), the PROTEIN PHOSPHATASE 2Cs (PP2Cs), and SNF1-RELATED PROTEIN KINASE 2s (SnRK2s) (Cutler *et al.*, 2010). The PYR/RCARs are the ABA receptors, the PP2Cs [e.g. the *ABA INSENSITIVE 1* (*ABI1*) gene] act as negative regulators of the pathway, and the SnRK2s act as positive regulators of downstream signalling (Ma *et al.*, 2009; Park *et al.*, 2009).

ABA-deficient mutants *aba1* and *aba2* display a general delay in flowering in LDs, which is more evident under drought conditions as well as reduced florigen transcript accumulation. Similar to *aba1*, mutants of *GI* are impaired in DE, and display no florigen up-regulation under drought conditions (Riboni *et al.*, 2013). The nature of GI signalling upstream of the florigen activation during DE is however unclear. Because no DE occurs in wild-type plants under SDs, one can conclude that GI activates DE by mediating photoperiodic signals. However, such a mechanism does not appear to require CO activity, since *co* mutants display a normal DE response (Han *et al.*, 2013; Riboni *et al.*, 2013). Modes of GI-dependent but CO-independent pathways include the activation of a class of miRNA, the *miR172*, which targets the *APETALA 2*-like factors that repress *FT* and other flowering genes (Jung *et al.*, 2007; Mathieu *et al.*, 2009). The role of GI in DE may also be indirect and/or biochemically distinct from its role in photoperiodic flowering. For example, GI affects phytochrome signalling (Huq *et al.*, 2000; Martin-Tryon *et al.*, 2007; Oliverio *et al.*, 2007), clock function (Park *et al.*, 1999; Fowler *et al.*, 1999; Mizoguchi *et al.*, 2005), and several plant–environment responses, namely salinity and freezing tolerance (Han *et al.*, 2013; Kim *et al.*, 2013*b*; Fornara *et al.*, 2015; Xie *et al.*, 2015), through mechanisms which cannot be fully ascribed to the canonical photoperiodic signalling cascade.

In this study, tests were carried out to elucidate the role of GI signalling in the DE response. We analysed the DE response and patterns of florigen accumulation in Arabidopsis mutant backgrounds with varying levels of *CO* and in the presence or absence of GI. To assess the role of ABA in the GI-mediated pathway, we combined mutants impaired in ABA signalling with transgenic plants overexpressing *GI*. We show that impaired ABA signalling affects GI downstream functions and/or activity, thus causing reduced accumulation of florigen genes, but no effects on *CO* accumulation. Our results also clarify the relationship between GI and CO in the context of DE response by showing that the drought/ABA-dependent activation of *FT* requires *CO*. In contrast, up-regulation of

TSF under drought stress can occur without CO, thus expanding the repertoire of regulatory mechanisms of florigen gene activation in plants. Alongside these results, we also demonstrate a florigen-independent floral repressive role for ABA in flowering, which requires *SOC1*. The transition to flowering under drought conditions thus depends on activation of separate ABA-dependent developmental programmes.

Materials and methods

Plant materials and growing conditions

In this study, we used wild-type *Arabidopsis thaliana* plants, ecotype Columbia (Col-0) or Landsberg *erecta* (L*er*). Mutant or transgenic lines were obtained from the Nottingham Arabidopsis Stock Centre or other laboratories as detailed in Supplementary Table S3 at *JXB* online. Seeds were stratified in the dark at 4 °C for 2 d before sowing, and plants grown in a controlled-environment cabinet at a temperature of 18–23 °C, 65% relative humidity, under either LD (16 h light/8 h dark) or SD (8 h light/16 h dark) photoperiods. Light was provided by cool white fluorescent tubes (Philips Lighting, F36W/33-640 36W) at a fluence of 120–150 µmol m^{-2} s^{-1} (photosynthetically active radiation). The procedures to impose drought stress, and perform photoperiod shift experiments were previously detailed (Riboni *et al.*, 2013).

Experiments in Fig. 1B were performed in a greenhouse, with a semi-controlled climate. Temperature was 19–23 °C and relative humidity was set at 65%. Natural light was supplemented by incandescent (metal halide) lamps when external light was <150 µmol m^{-2} s^{-1} (photosynthetically active radiation) in an LD photo cycle. Two independent greenhouse experiments were performed (autumn 2015 in Milan). ABA application experiments were performed by daily supplying 2 ml of ABA (25 µM) or mock solutions (0.025% v/v ethanol) 7 h after dawn. ABA applications started 3 d after germination and continued for 21 d. Each Arabasket pot was fitted with a pipette tip to facilitate the application of the solutions directly in the soil and thus in contact with roots (Supplementary Fig. S1).

Isolation of double mutants and genotyping

Mutant combinations were generated by crossing. The *aba1-6* mutation was genotyped as described in Riboni *et al.* (2013). *ft-10* mutants were selected on Murashige and Skoog plates containing Sulafadiazide as described (Rosso *et al.*, 2003). *abi1-1* mutants were selected by genomic PCR amplification with primers flanking the *abi1-1* polymorphism followed by digestion with *Nco*I. Genotyping primers for *tsf1-1*, *co-10*, and *abi1-1* are listed in Supplementary Table S4. Plants carrying the *gi-2* and *soc1-1* alleles were selected based on their late flowering phenotype, while *elf3-1* mutants were selected on the basis of their early flowering and long hypocotyl.

RNA extraction and real-time qPCR

Total RNA was extracted with TRIzol reagent (Invitrogen). A 1.5 µg aliquot of total RNA was used for cDNA synthesis with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR was performed as previously detailed (Riboni *et al.*, 2013) and PCR primers are provided in Supplementary Table S4.

Molecular cloning and plant transformation

A 2.2 kbp promoter region upstream of the *ABI1* coding sequence was cloned using the Gateway cloning technology (Invitrogen) with specifics primers (Supplementary Table S4). The promoter was cloned into the pDONR207 entry vector (Invitrogen) and moved into the pBGWFS7 destination vector (Karimi *et al.*, 2002). The resulting plasmid was introduced into *Agrobacterium* strain GV3101 (pMP90RK) (Koncz and Schell, 1986) and transformed in wild-type Col-0 plants by floral dip (Clough and Bent, 1998). Six independent transgenic plants were selected based on the segregation of Basta resistance in a Mendellian 3:1 ratio in the T_2 generation and analysed for β-glucuronidase (GUS) staining.

GUS assay

Plants were grown under LDs and sampled at the indicated Zeitgeber time (ZT) time. Tissue was fixed for 30 min at 0 °C with 90% (v/v) acetone. After being washed in 50 mM sodium phosphate buffer, pH 7.0 they were incubated for 14 h at 37 °C in staining solution [0.5 mg ml^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% (v/v) Triton X-100]. Samples were cleared with a chloral hydrate:glycerol:water solution (8:1:2, v/v/v) for 3 h and then stored in 70% (v/v) ethanol before GUS histochemical reactions were visualized under a stereomicroscope.

Results

ABA promotes FT expression through CO

Mutants of *aba1-6* were later flowering compared with the wild type under LDs (Fig. 1A–C). We confirmed a similar late flowering phenotype in *aba2-1* mutants, defective in the final steps of ABA biosynthesis (Finkelstein, 2013). Soil applications of ABA could accelerate flowering in wild-type plants, reminiscent of DE response (Fig. 1A; Supplementary Table S1) (Koops *et al.*, 2011). Using this experimental set-up, we could also largely rescue the late flowering of *aba1-6* and *aba2-1* mutants, indicating a role for ABA as an activator of flowering (Fig. 1A, B).

We have previously demonstrated that ABA activates flowering under LDs but not SDs and that ABA affects photoperiodic signalling upstream of *FT* expression (Riboni *et al.*, 2013). To understand how ABA interacts with photoperiod signalling to affect flowering, we generated combinations of ABA-deficient (*aba1-6*) and photoperiodic pathway mutants (Fig. 1C, D; Supplementary Table S1). Consistent with lack of flowering defects of *aba1-6* under SDs (Riboni *et al.*, 2013), double mutants of *gi-2 aba1-6* displayed a similar flowering time compared with *gi-2* single mutants under LDs (Fig. 1C, F). Since double mutants of *ft-10 aba1-6* were later flowering than *ft-10* single mutants, ABA could affect flowering time via other florigen genes, namely *TSF* (Fig. 1C, F). The *tsf-1 ft-10 aba1-6* triple mutants were slightly later flowering than *tsf-1 ft-10* double mutants (Fig. 1C, F). *TSF* thus contributes to the late flowering phenotype of *ft-10 aba1-6* plants although ABA also appears to have an effect on other floral pathways, independent of *FT* and *TSF*. Interestingly, double mutants of *co-10 aba1-6* were similar to *co-10* single mutants, indicating that *CO* is also required for the late flowering phenotype of *aba1-6* mutants (Fig. 1D).

Unlike *gi*, *co* mutants generate a DE response, indicating that high levels of ABA accumulation, as a result of drought stress, may eventually overcome CO function to activate flowering (Riboni *et al.*, 2013). To test whether drought could activate the florigen genes in the absence of a functional CO protein we grew wild-type and *co-10* mutant plants under

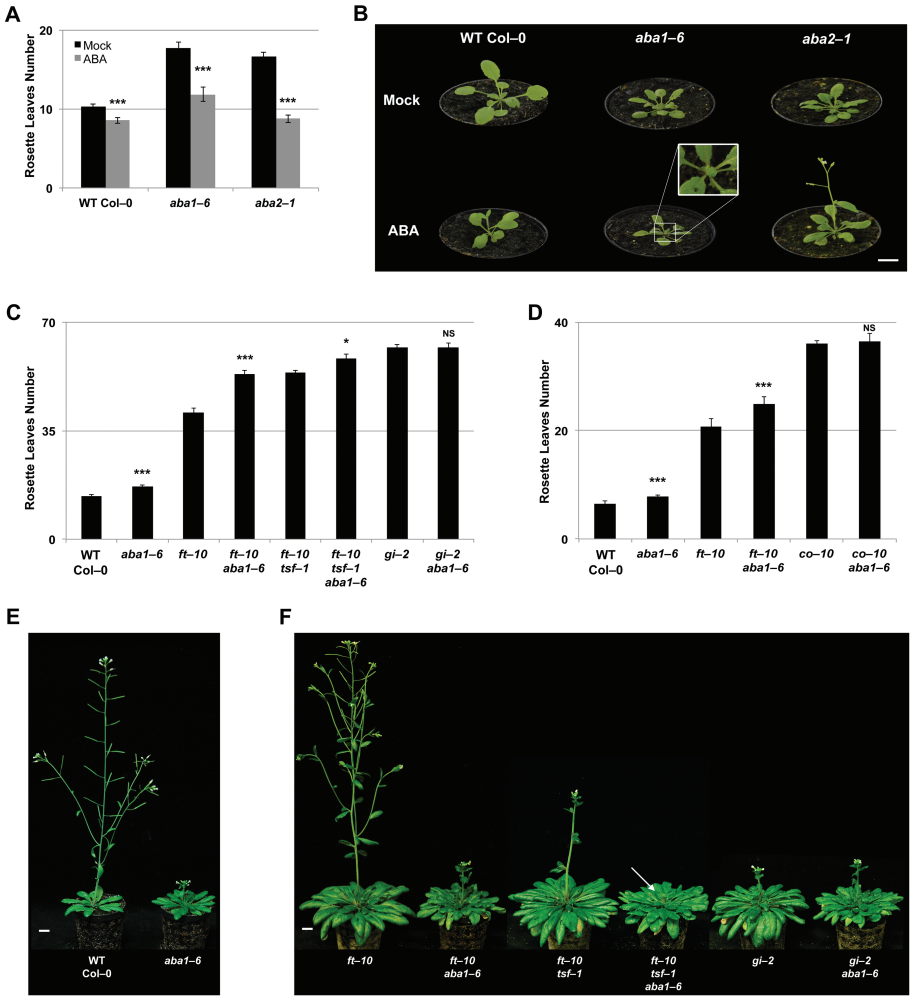

Fig. 1. ABA activates flowering through GI, CO, and the florigen genes. (A) Mean number of rosette leaves of the wild type (Col-0) and ABA-deficient mutant plants grown under LDs and treated with ABA or mock treated. Error bars represent ±SE, *n*=15. Student's *t*-test *P*-values ≤0.001 (***) compared with mock treatment. (B) Images of representative 24-day-old plants of the indicated genotypes grown under LDs and treated with ABA or mock treated. Inset of *aba1-6* shows a visible inflorescence. (C and D) Mean number of rosette leaves of the wild type (Col-0) and flowering time mutants grown under LDs. Error bars represent ±SE, *n*=15. Student's *t*-test *P*-values ≤0.05 (*), ≤0.001 (***), >0.05 not significant (NS) are shown to indicate differences between mutants and the corresponding mutant containing the *aba1-6* allele. The experiment in (D) was performed under semi-controlled greenhouse conditions. (E) and (F), Images of representative plants of the indicated genotypes grown under LDs. (E) Wild-type Col-0 and *aba1-6* mutant plants are 4 weeks old, (F) *ft-10*, *ft-10 aba1-6*, *ft-10 tsf-1*, *ft-10 tsf-1 aba1-6*, *gi-2*, and *gi-2 aba1-6* mutant plants are 14 weeks old. The arrow indicates the visible bolt in *ft-10 tsf-1 aba1-6*. Scale bars=1 cm. (This figure is available in colour at *JXB* online.)

control or water stress conditions in SDs before shifting to LDs to induce a photoperiodic response. As expected, in wild-type plants *FT* expression was strongly up-regulated during the photo-extension period and even further increased under low watering conditions (Fig. 2A). In the *co-10* mutants, the levels of *FT* transcripts were barely detectable at any time point, independent of the watering regime, indicating that drought stress cannot cause *FT* up-regulation in the absence of a functional *CO* (Fig. 2B). The pattern of accumulation of *TSF* showed diurnal oscillations similar to those of *FT* in wild-type plants, peaking at dusk during the photo-extension period (Fig. 2A, B). Similar to *FT*, *TSF* expression was increased in coincidence with the photo-extension period under drought conditions. Furthermore in *co-10* mutants, *TSF* levels were much lower compared with the wild type under normal watering conditions, confirming a role for *CO* in *TSF* transcriptional activation (Yamaguchi *et al.*, 2005; Jang *et al.*, 2009). Surprisingly, drought stress

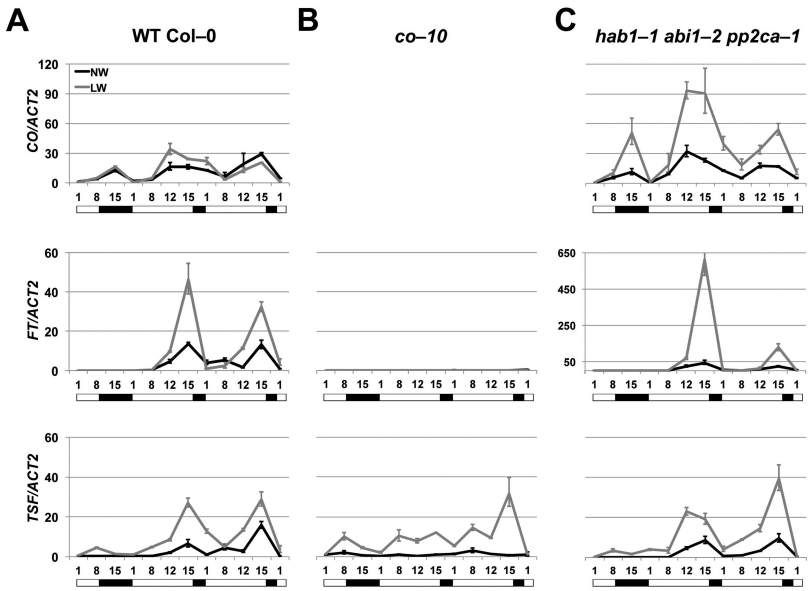

Fig. 2. CO is required for the activation of FT under drought stress. (A–C) Real-time qPCR of *CO*, *FT*, and *TSF* transcripts in 3-week-old wild-type (Col-0) (A), *co-10*, (B) and *hab1-1 abi1-2 pp2ca-1* (C) seedlings. Plants were subject to normal watering (NW; black lines) or low watering (LW; grey lines) regimes and harvested at the indicated time points in coincidence with the light phase (open bar) or in the dark (black bar) during an SD to LD shift. At each time point, values represent fold change variations of *CO*, *FT*, and *TSF* transcript levels relative to Col-0 under NW. *ACT2* expression was used for normalization; error bars represent the SD of two technical replicates. A representative experiment of two biological replicates is shown.

caused *TSF* up-regulation in the *co-10* background, partially resuming its diurnal cycle with peaks at ZT8 under the SD part of the experiment and at ZT15 following a photo-extension. Slightly increased *TSF* levels were observed during SDs under drought conditions (on average 3.8 ± 1.6-fold) but this was not correlated with a DE phenotype under SDs in *co-10* mutants (Fig. 2B, D). Thus, unlike *FT*, *TSF* can be up-regulated under drought conditions in a *CO*-independent manner.

GI is required for DE downstream of CO transcriptional activation

Our experiments indicate that ABA promotes *FT* transcript accumulation through *CO*. However, *CO* transcript levels are not greatly affected by drought stress or when ABA level are reduced (Han *et al.*, 2013; Riboni *et al.*, 2014). Here we wanted to test whether drought could affect flowering downstream of *CO* transcriptional activation events, by analysing mutants of *cdf1-R cdf2-1 cdf3-1 cdf5-1*, hereafter referred to as *cdf1235*, characterized by constitutively elevated *CO* levels (Fornara *et al.*, 2009). The *cdf1235* mutants flowered early and produced a DE response quantitatively similar to that of the wild type under LDs (Fig. 3A). Despite their early flowering phenotype under SDs, *cdf1235* plants did not produce any DE response (Fig. 3B), suggesting a requirement for LDs in DE response, even when *CO* levels are elevated (Fornara *et al.*, 2009) (Fig. 3C). We therefore compared the flowering time and DE response of the quadruple *cdf1235* mutant with that of *gi cdf1235* quintuple mutants under LDs (Fig. 3A). As previously shown, mutants of *cdf1235* are slightly earlier flowering than *gi cdf1235* under normal watering conditions (Fornara *et al.*, 2009). However, while the *cdf1235* mutants produced a DE response, the *gi cdf1235* did not (Fig. 3A). We next sought to ascertain if the lack of DE response in the *gi cdf1235* mutants was correlated with impaired transcriptional up-regulation of the florigen genes under drought stress. Control and water-stressed wild-type, *cdf1235* and *gi cdf1235* plants were grown under SD conditions for 2 weeks before shifting to LDs, and transcript levels were analysed at ZT8 (corresponding to dusk in the SDs) and ZT12 (4 h after the photo-extension) (Fig. 3C–E). As expected, the levels of *CO* transcript were generally higher in *cdf1235* and *gi cdf1235* mutants as compared with the wild type. Under drought conditions, we observed a small increase in *CO* transcript abundance in all the genotypes analysed at any time point, suggesting a contribution of drought stress in *CO* transcript accumulation (Fig. 3C). We finally determined how different patterns of *CO* transcript were correlated with accumulation of florigen genes (Fig. 3D, E). Under well-watered conditions, mutants of *cdf1235* showed the largest *FT* and *TSF* transcript accumulations before and after the photo-extension period. Mutants of *gi cdf1235* displayed levels of *FT* and *TSF* intermediate between the wild type and the *cdf1235* mutants. This is probably as a result of residual CDF-mediated repression in *cdf1235* on both *CO* and *FT* promoters (Fornara *et al.*, 2009; Song *et al.*, 2012). However, while both the wild type and the *cdf1235* mutants showed a significant and similar up-regulation of *FT* and *TSF* under drought stress conditions

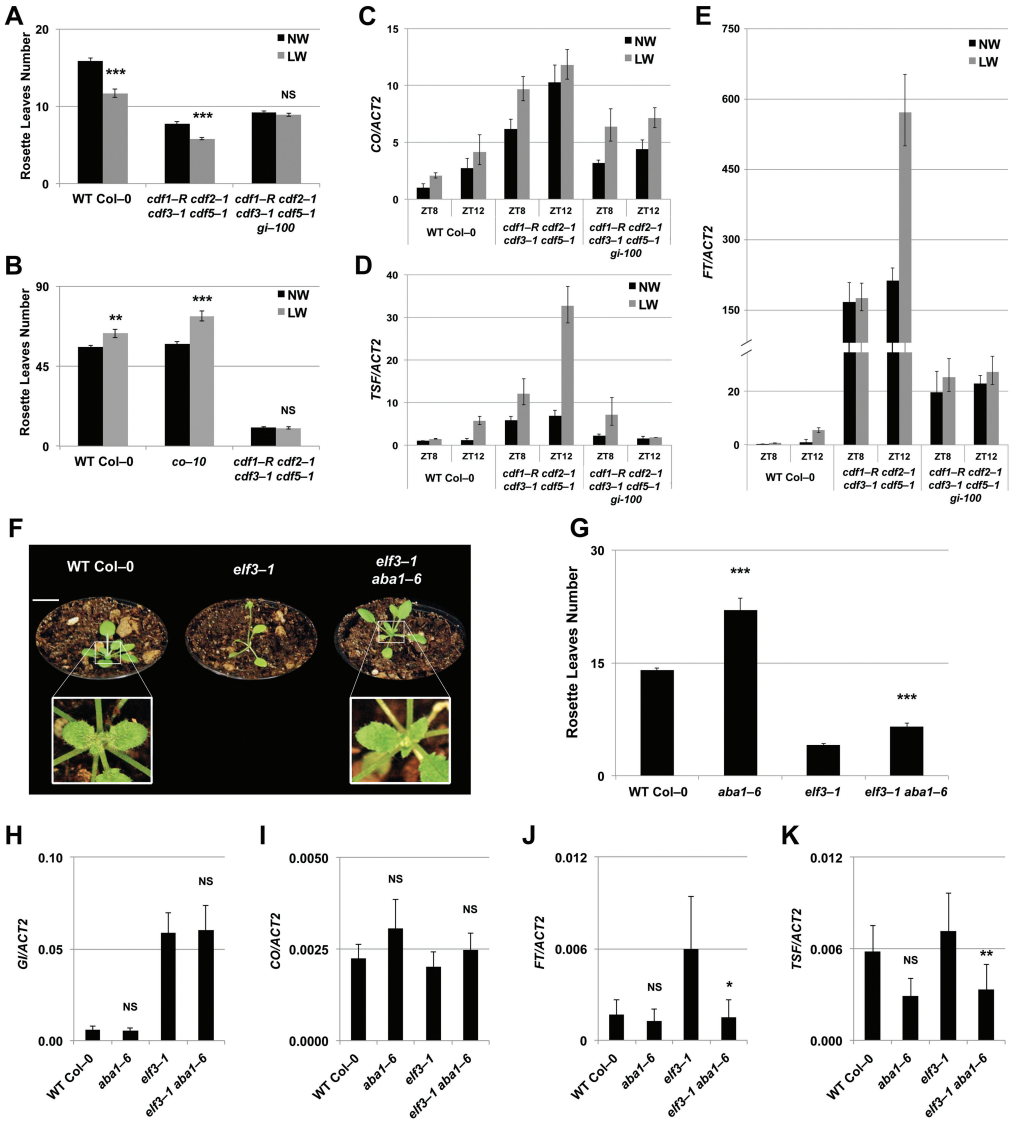

Fig. 3. ABA promotes GI and CO functions to activate the florigen genes. (A and B) Mean number of rosette leaves of the wild type (Col-0) and flowering time mutants subject to normal watering (NW; black bars) or low watering (LW; grey bars) regimes grown under LDs (A) and SDs (B). Error bars represent ±SE *n*=15. Student's t-test *P* values ≤0.001 (***), >0.05 not significant (NS) compared with NW. (C–E) Real-time qPCR of *CO* (C), *TSF* (D), and *FT* (E) transcripts in 2-week-old wild-type (Col-0), *cdf1-R cdf2-1 cdf3-1 cdf5-1*, and *cdf1-R cdf2-1 cdf3-1 cdf5-1 gi-100* seedlings. Plants were subject to NW (black columns) or LW (grey columns) regimes and harvested at the indicated Zeitgeber time during a shift from SDs to LDs. ZT8 represents dusk in SDs and ZT12 represents 4 h of photo-extension. At each time point, values represent fold change variations of *CO*, *FT*, and *TSF* transcript levels relative to the wild type at ZT8 under NW. *ACT2* expression was used for normalization; error bars represent the SD of two technical replicates. A representative experiment of two biological replicates is shown. (F) Images of representative plants grown under LDs for 27 d. Insets shows a visible inflorescence in *elf3-1 aba1-6* double mutants, which is not visible in the wild type. (G) Mean numbers of rosette leaves of the wild type (Col-0) and mutants under LDs. Error bars represent ±SE, *n*=5–12. Student's *t*-test *P*-values ≤0.001 (***) are shown to indicate differences between mutants and the corresponding mutant containing the *aba1-6* allele. (H–K) Real-time qPCR of *GI* (H), *CO* (I), *FT* (J), and *TSF* (K) transcripts in 12-day-old seedlings grown under LDs and sampled at ZT16. Data shown are from 5–6 biological replicates. Error bars represent ±SD. Differences between the wild type versus *aba1-6* and *elf3-1* versus *elf3-1 aba1-6* double mutants are here highlighted with *P*-values ≤0.01 (**), ≤0.05 (*), >0.05 not significant (NS), one-way ANOVA with Tukey's HSD (honestly significant difference) test. (This figure is available in colour at *JXB* online.)

at ZT12 (2- to 4-fold, respectively), no such up-regulation occurred in the *gi cdf1235* mutants (Fig. 3D, E).

In a complementary approach, we asked whether ABA production might be required for *FT* transcriptional activation when *GI* levels are increased. Mutants of *early flowering 3* (*elf3*) are extremely early flowering, accumulate high levels of *FT*, and present increased accumulation of *GI* transcript and GI protein (Fowler *et al.*, 1999; Kim *et al.*, 2005; Yu *et al.*, 2008). This early flowering phenotype requires ABA since *elf3-1 aba1-6* double mutants were significantly later flowering compared with *elf3-1* single mutants (Fig. 3F, G). *FT* and *TSF* transcript levels were slightly but not significantly reduced in *aba1-6* mutants compared with the wild type at this early developmental stage (Fig. 3J, K; Supplementary Table S2). However, double mutants of *elf3-1 aba1-6* had a significant reduction in both *FT* and *TSF* levels compared with the *elf3-1* single mutants (Fig. 3J, K; Supplementary Table S2). The reduced levels of *FT* and *TSF* in *elf3-1 aba1-6* compared with *elf3-1* mutants were not caused by diminished *GI* or *CO* transcript accumulations (Fig. 3H, I; Supplementary Table S2), indicating that ABA might be required for the activation of GI and CO signalling.

ABA signalling genes control FT transcript accumulation with little effect on CO

We analysed ABA-hypersensitive mutants plants *hab1-1 abi1-2 pp2ca-1*, impaired in three ABA-related PP2C phosphate genes, under different watering and photoperiodic conditions (Rubio *et al.*, 2009). Consistent with previous observations, mutants of *hab1-1 abi1-2 pp2ca-1* had much increased (up to 6-fold) levels of *FT* compared with the wild type under LDs (Riboni *et al.*, 2013) (Fig. 2C). The experiment in Fig. 2C also shows that *FT* expression was even further activated under drought conditions compared with the wild type (up to 13.3-fold). In contrast, *TSF* expression was not clearly increased in *hab1-1 abi1-2 pp2ca-1* plants compared with the wild type under any watering condition. No *FT* or *TSF* up-regulation occurred under SDs in the *hab1-1 abi1-2 pp2ca-1* mutants under any watering condition.

Under control conditions the strong up-regulation of *FT* in *hab1-1 abi1-2 pp2ca-1* plants was not caused by increased *CO* levels, which were comparable with those observed in the wild type (Fig. 2C). Increased levels of *CO* were, however, observed in the *hab1-1 abi1-2 pp2ca-1* mutants under drought stress, indicating that high levels of ABA signalling can ultimately induce the transcriptional activation of *CO* (Koops *et al.*, 2011; Yoshida *et al.*, 2014).

To explore further the role of ABA signalling in the transcriptional control of *FT*, we analysed *abi1-1* mutant plants (L*er* background), carrying a dominant mutation in the *PP2C* phosphatase *ABI1* (Koornneef *et al.*, 1984) which results in severely reduced ABA responses. *abi1-1* mutant plants did not show flowering defects under LDs, but exhibited an early flowering phenotype under SDs, consistent with previous observations (Martínez-Zapater *et al.*, 1994; Chandler *et al.*, 2000) (Fig. 4A, B). Ruling out an ecotype-specific effect for ABA action in flowering, the ABA biosynthetic mutants *aba1-1* and *aba1-3* (L*er* background) showed a marginal late flowering phenotype compared with the wild type under LDs (ANOVA $P<0.01$ and $P<0.05$, respectively), but no defects under SDs (Fig. 4A, B). The late flowering phenotype of these *aba1* mutants was more pronounced under drought conditions and LDs, indicative of a reduced DE response (Fig. 4A). Mutants of *abi1-1* were even more impaired in the DE response compared with the *aba1* alleles, producing on average 14 ± 2% more leaves ($n = 8$ independent experiments, 15 plants each), relative to the untreated control.

We next analysed the pattern of accumulation of the florigen genes in *abi1-1* plants. As expected, in wild-type plants, the accumulation of *FT* was strongly induced under drought conditions in a photoperiod-dependent manner (Fig. 5A). *TSF* expression was instead down-regulated under drought conditions in the L*er* background, revealing an ecotype-specific effect for *TSF* regulation under drought (Fig. 5A). Lower levels of *FT* and *TSF* were observed in the *aba1-1* mutants compared with the wild type under both normal watering (*TSF*) and drought conditions (*FT* and *TSF*), confirming the contribution of ABA in both *FT* and *TSF* regulation (Fig. 5B) (Riboni *et al.*, 2013). Strikingly, in *abi1-1* plants the levels of *FT* were dramatically reduced compared not only with the wild type but also with *aba1-1* plants, under any watering condition analysed (Fig. 5C). Such low expression of the florigen genes did not depend on reduced *CO* transcript accumulation in *abi1-1* which was, if anything, up-regulated (Fig. 5C). Taken together, our data point to a model where ABA affects accumulation of florigen genes without an effect on *CO* expression.

Loss of PP2C function (as in *hab1-1 abi1-2 pp2ca-1*) results in increased *FT* transcript accumulation, while expression of a gain-of-function form of *ABI1* (as in *abi1-1*) leads to reduced *FT* activation. To determine whether the negative regulation of *ABI1* on *FT* expression could be exerted in the cells expressing *FT*, we fused a 2.2 kb promoter region of *ABI1* to the GUS reporter. We detected GUS staining in several independent transgenic T_2 plants ($n = 6$) with comparable results, at ZT8, where *ABI1* transcript accumulation is highly abundant according to a publicly available data set (http://diurnal.mocklerlab.org; Mockler *et al.*, 2007). For comparison, we also studied the pattern of GUS activity in Arabidopsis transgenic lines marking the *FT* expression domain; the *ABA2* (Lin *et al.*, 2006; Kuromori *et al.*, 2014) and the *FT* promoter itself (Notaguchi *et al.*, 2008). Histochemical detection in young seedlings revealed that *ABI1* expression (Fig. 4E) occurred in the vasculature of cotyledons in a pattern similar to *ABA2* and *FT* (Fig. 4C, D), demonstrating an overlap between ABA biosynthesis and signalling genes in the tissue known to be the source of FT protein production. Broadly distributed GUS staining was also observed in the apical region of *ABI1::GUS* transgenic plants (Fig. 4H). This pattern of expression may also indicate a role for ABA signalling in the shoot apex.

Impaired ABA signalling negatively affects GI action

Whether impairing ABA signalling affects GI action was tested by generating *abi1-1 35S::GI* plants. As previously

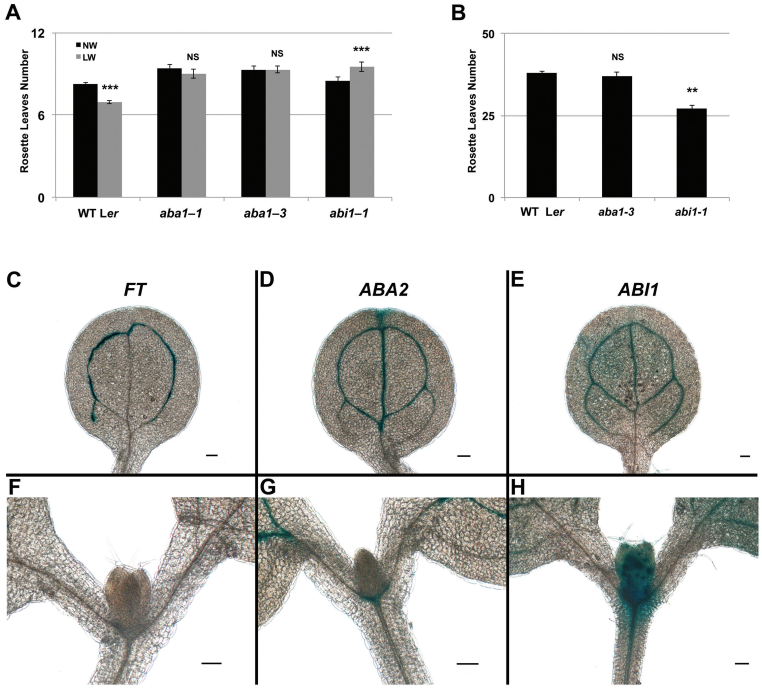

Fig. 4. A negative role for ABA in flowering. (A and B) Mean number of rosette leaves of the wild type (L*er*) and ABA-deficient or signalling mutants grown under LDs and subject to normal watering (NW; black bars) or low watering (LW; grey bars) regimes (A), or under SDs in NW regime (B). Error bars represent ±SE n=15. Student's t-test P-values ≤0.001 (***), >0.05 not significant (NS), compared with NW (A). One-way ANOVA with Tukey's HSD (honestly significant difference) test P-values ≤0.01 (**), >0.05 not significant (NS), compared with the wild type (B). (C–H) Histochemical GUS detection in transgenic seedlings expressing *pFT::GUS* (C) and (F), *pABA2::GUS* (D) and (G), and *pABI1::GUS* (line # 1) (E) and (H) in the Col-0 background, scale bars=100 μm. (This figure is available in colour at *JXB* online.)

observed, *35S::GI* plants had increased levels of *FT* under both SDs and LDs compared with the wild type (Mizoguchi *et al.*, 2005). Under drought conditions, *FT* expression was generally less responsive to drought in the *35S::GI* background compared with the wild type (Fig. 5D). The levels of *TSF* were much more increased in *35S::GI* plants compared with the wild type during the SD part of the experiment. However, no further up-regulation of *TSF* occurred as a result of drought stress compared with normal watering (Fig. 5D). The overaccumulation of *FT* observed in *35S::GI* plants was strongly rescued in the *abi1-1 35S::GI* mutants under any watering conditions (Fig. 5E). The levels of *TSF* transcript fell even more severely in *abi1-1 35S::GI* plants compared with *35S::GI*. Such reductions in florigen accumulation in *abi1-1 35S::GI* plants were not related to decreased *CO* levels as these were much higher than in the wild type (Fig. 5A, E). Interestingly the levels of *CO* in *abi1-1 35S::GI* plants were only mildly reduced compared with *35S::GI*, which could suggest that the negative role exerted by abi1-1 protein on GI signalling is more related to *FT* and *TSF* regulation rather than to *CO* (Fig. 5D, E).

Our data describe a regulatory role of ABA in GI signalling. Such ABA-mediated post-transcriptional activation of *GI* is consistent with previous observations on *35S::GI* plants showing a DE-responsive phenotype under SDs (Riboni *et al.*, 2013). In contrast, no DE response occurred in *abi1-1 35S::GI* mutants, which flowered much later compared with well-watered plants of the same genotype, although still earlier than *abi1-1* plants (Fig. 5F). Under normal watering conditions, double mutants of *abi1-1 35S::GI* had a similar flowering phenotype to *35S::GI* plants, despite showing reduced accumulation of the florigen genes (Fig. 5E, F). A similar observation could be made for *abi1-1* plants, which did not show flowering defects under LDs compared with the wild type, but had reduced florigen expression (Fig. 5A, C). We conclude that late flowering of *abi1-1* or *abi1-1 35S::GI* plants under drought stress cannot be solely ascribed to reduced florigen up-regulation.

A negative role for ABA signalling in flowering

The early flowering of *abi1-1* plants under SDs (Fig. 4B) implies that ABA signalling also exerts a negative role in flowering, which is usually undetectable under LDs or in ABA biosynthetic mutants (Fig. 4A). Supporting this model, we have previously reported a delay of flowering time under SDs in mutants of *hab1-1 abi1-2 pp2ca-1* and observed a similar phenotype also in *hab1-1 abi1-2 abi2-2*

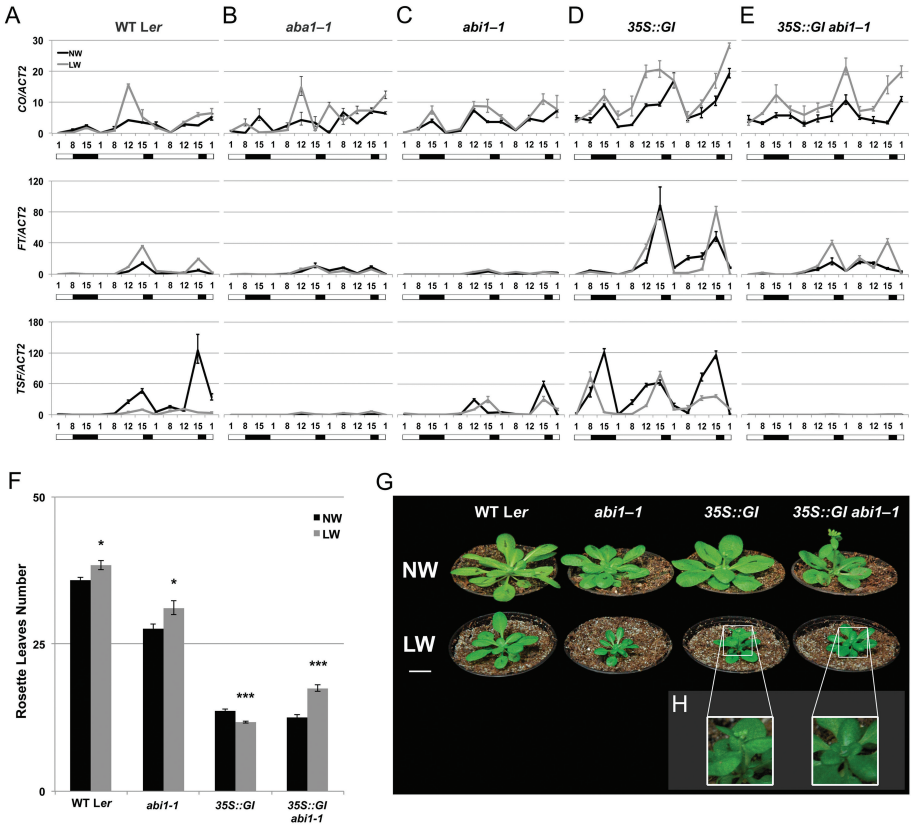

Fig. 5. ABA activates GI signalling and florigen expression with little effect on CO transcript accumulation. (A–E) Real-time qPCR of *CO*, *FT*, and *TSF* transcripts in 2-week-old wild-type (*Ler*) (A), *aba1-1* (B), *abi1-1* (C), *35S::GI* (D), and *35S::GI abi1-1* (E) seedlings. Plants were subject to normal watering (NW; black lines) or low watering (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 an SD to LD shift. At each time point, values represent fold change variations of *CO*, *FT*, and *TSF* transcript levels relative to *Ler* under NW. *ACT2* expression was used for normalization; error bars represent the SD of two technical replicates. A representative experiment of two biological replicates is shown. (F) Mean number of rosette leaves of the wild type (*Ler*) and mutants grown under SDs and subject to NW (black bars) or LW (grey bars) regimes, Error bars represent ±SE n=15. Student's t-test P-values ≤0.05 (*), ≤0.001 (***) compared with NW. (G) Images of representative 5-week-old plants of the indicated genotypes grown under SDs and subject to NW or LW regimes. Scale bar=1 cm. (H) Higher magnification of LW *35S::GI* and *35S::GI abi1-1* plants shown in (G). Note the appearance of a bolt in *35S::GI* but not in *35S::GI abi1-1*.

plants (Riboni *et al.*, 2013) (Supplementary Fig. S2). *abi1-1* mutants showed no increase in *FT* and *TSF* levels under SDs (Fig. 5B). In contrast, the accumulation of another floral integrator, *SOC1*, was increased in *abi1-1* plants as compared with the wild type under any photoperiodic condition (Fig. 6A). Mutants of *abi1-1* also had strongly reduced levels of *FLOWERING LOCUS C* (*FLC*) (Fig. 6B), a transcriptional repressor of *SOC1* which contributes to delaying flowering under drought condition (Riboni *et al.*, 2013; Y. Wang *et al.*, 2013; Shu *et al.*, 2016). Since *SOC1* integrates different floral pathways in the SAM (Moon *et al.*, 2003; Wang *et al.*, 2009; Song *et al.*, 2012, 2014) which promote flowering under SDs we created the *abi1-1 soc1-1* double mutants. Under SDs, these plants displayed a flowering time similar to the *soc1-1* single mutants. With respect to flowering time, *soc1-1* is thus completely epistatic to *abi1-1*, indicating that *SOC1* activity is required for the early flowering of *abi1-1* mutants under SDs (Fig. 6C).

Under LDs, *abi1-1 soc1-1* double mutants were later flowering than *soc1-1* single mutants (Fig. 6D). Thus, the knocking out of *SOC1* produces a novel flowering phenotype in the *abi1-1* background, consistent with ABA being able to affect flowering differentially in different domains of the plant; by promoting *FT* expression in the leaves and negatively regulating floral stimuli in the SAM (Fig. 6E).

Discussion

A fundamental question related to the DE mechanism is how ABA signals are integrated in the photoperiodic flowering network. Here we provide evidence for how ABA controls *FT* gene expression under normal and drought stress

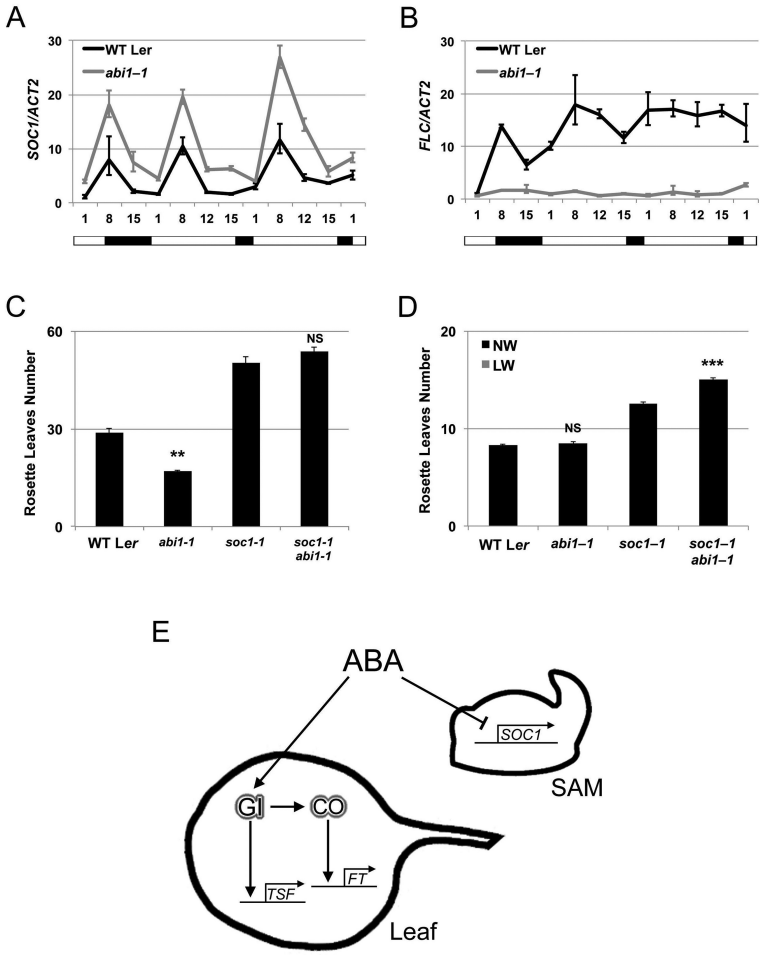

Fig. 6. The inhibitory role of ABA in flowering requires SOC1. (A and B) Real-time qPCR of *SOC1* (A) and *FLC* (B) transcripts in 2-week-old wild-type (L*er*) and *abi1-1* seedlings. The experimental conditions were described in Fig. 5. *ACT2* expression was used for normalization; error bars represent the SD of two technical replicates. A representative experiment of two biological replicates is shown. (C and D) Mean number of rosette leaves of the wild type (L*er*) and mutants grown under SDs (C) or LDs (D). Error bars represent ±SE *n*=15. Differences between the wild type versus *abi1-1* and *soc1-1* versus *soc1-1 abi1-1* double mutants are here highlighted with *P*-values ≤0.001 (***), ≤0.01 (**), >0.05 not significant (NS), one-way ANOVA with Tukey's HSD (honestly significant difference) test. (E) Model summarizing the proposed modes of ABA action in flowering. In the leaves, under LDs, drought promotes ABA accumulation leading to enhanced GI signalling and activation of florigen genes. CO protein is required for *FT* up-regulation, but not *TSF*. At the same time, at the shoot apex ABA represses flowering, downplaying *SOC1* signalling, independent of photoperiodic conditions.

conditions by affecting photoperiodic signalling. We also highlight a negative effect of ABA during the floral transition of Arabidopsis, which is independent of the photoperiodic pathway.

ABA requires both GI and CO to regulate FT

Our genetic data point to a model where ABA requires both *GI* and *CO* to affect flowering under LDs through the transcriptional activation of the florigen genes. Since mutants of *ft-10 tsf-1 aba1-6* were still slightly later flowering than *ft-10 tsf-1*, it is possible that ABA may act on other pathways or through activation of *MFT*, a third florigen gene with a marginal role in flowering (Kim *et al.*, 2013*a*).

Expression and phenotypic analyses of *cdf1235*, *gi cdf1235*, as well as *aba1 elf3* mutants collectively suggest that ABA promotes GI and CO signalling upstream of the florigen genes. CO function is essential for the drought-dependent activation of *FT* (but not *TSF*) as demonstrated by the lack of *FT* accumulation in *co* mutants under drought conditions. Therefore, although we could not resolve the underlying molecular mechanism, our data underscore a regulatory role

for ABA in stimulating photoperiodic signalling. In further support of this model, *35S::GI* plants under SDs generate a DE response, suggesting drought/ABA acting independently of *GI* transcript accumulation. Secondly, we observe a strong reduction in accumulation of florigen transcripts in *abi1-1 35S::GI* compared with *35S::GI* plants. Thirdly, the pattern of *CO* accumulation in *abi1-1* or *abi1-1 35S::GI* plants is unaltered compared with their respective controls, as opposed to the florigen levels, which are very low. In the light of our results, abi1-1 protein appears to affect a specific aspect of GI function (the activation of *FT*) without producing significant effects on the transcriptional profile of *CO* accumulation. Previous studies have demonstrated genetically separable roles for GI in regulating the circadian clock and flowering (Mizoguchi *et al.*, 2005; Martin-Tryon *et al.*, 2007) which could reflect distinct biochemical activities for GI in these two pathways. ABA might thus control a novel biochemical function of GI.

GI is found at different promoter locations of *FT* in association with transcriptional repressors including SHORT VEGETATIVE PHASE and TEMPRANILLO (Sawa and Kay, 2011). A substrate of the GI–FKF1 complex, CDF1, also binds to the *FT* promoter and acts as a transcriptional repressor (Sawa *et al.*, 2007). Furthermore, by activating *miR172* expression, *GI* directs post-transcriptional gene silencing of the *AP2*-type transcriptional repressors of *FT* (Jung *et al.*, 2007). Overexpression of a *miR172*-related miRNA of soybean facilitates the DE response, promotes *FT* accumulation under drought conditions, and increases ABA sensitivity of Arabidopsis (Li *et al.*, 2016). Thus, one role of GI could be to favour the recruitment of CO at the *FT* promoter by promoting the proteasome-dependent degradation or the post-transcriptional gene silencing of transcriptional repressors (such as *AP2-like*) in an ABA-dependent manner. Another, not mutually exclusive, model is that the combined presence of GI and ABA alters the pattern of CO protein accumulation during the day through an unknown mechanism. In addition to these post-transcriptional effects, there is evidence for other layers of transcriptional regulation of *CO* exerted by drought/ABA (Fig. 2C) (Koops *et al.*, 2011; Ito *et al.*, 2012; P. Wang *et al.*, 2013; Yoshida *et al.*, 2014). The contribution of these regulatory nodes to DE will require further studies. Regardless of the mechanisms involved and considering the role of the circadian clock in the control of ABA accumulation and response (Fukushima *et al.*, 2009), our results suggest that daily variations in ABA signalling may represent a further layer of regulation of CO protein function.

Different modes of regulation of FT and TSF by drought

While *FT* and *TSF* share a common mechanism of transcriptional regulation through the photoperiodic pathway (Yamaguchi *et al.*, 2005; Jang *et al.*, 2009), they also display clear differences in their pattern of expression (Yamaguchi *et al.*, 2005), response to ambient temperature (Blázquez *et al.*, 2003), and other kinds of regulation (Michaels *et al.*, 2005; D'Aloia *et al.*, 2011; Liu *et al.*, 2014). In this work, we report variations in the transcriptional activations of *TSF* and *FT* in response to drought. Our expression studies on *co-10* mutants revealed that the expression of *TSF*, but not *FT*, is strongly induced by drought, even in the absence of functional CO. Previously we proposed a model whereby photoperiod-stimulated GI protein triggers a DE response via activation of the florigen genes, independent of *CO* (Riboni *et al.*, 2013). Based on our new results, this model only applies to *TSF* regulation, not *FT*. The DE response observed in the *co* mutants could therefore derive from residual *TSF* expression, which still depends on GI (Riboni *et al.*, 2013). Examples of *GI* acting independently of *CO* in activating the florigen genes have been described in the literature, but how these mechanisms are related to ABA signalling is unknown (Kim *et al.*, 2005; Mizoguchi *et al.*, 2005; Sawa and Kay, 2011). Other hormones modulate the expression of the florigen genes without an apparent contribution of *CO*. Cytokinin can induce the transcriptional activation of *TSF*, but not *FT*, irrespective of photoperiod conditions (D'Aloia *et al.*, 2011). Foliar applications of gibberellins under SDs promote flowering, at least in part through *FT* ad *TSF* and without a clear effect on *CO* transcript accumulation (Porri *et al.*, 2012). Similarly, there are examples of environmental cues activating *FT*, which do not fully require the activity of *CO* or *GI*, namely under elevated ambient temperature (Balasubramanian *et al.*, 2006). Here, we demonstrate that the activation of *TSF* can occur in the absence of *CO* under drought conditions but, unlike the previous examples, such activation requires *GI* (Riboni *et al.*, 2013).

Multiple and contrasting roles of ABA in flowering

The role of ABA during the floral transitions is controversial, as both positive and negative effects of ABA have been reported (Domagalska *et al.*, 2010; Conti *et al.*, 2014). Depending on the site of application, ABA exerts opposite roles in flowering. Unlike leaf applications, we show that root applications of ABA promote flowering, consistent with previous data (Koops *et al.*, 2011). Also, this treatment largely rescues the late flowering of ABA biosynthetic mutants. In the light of these results, root applications fully mimic the positive role of endogenous ABA in flowering.

Impairing the function of ABA-activated kinases SnRK2.2/2.3/2.6 results in early flowering, especially under SDs, supporting a negative role for ABA in flowering (P. Wang *et al.*, 2013). Arguing against a direct negative role of the SnRK2s in the flowering network, overexpression of *SnRK2.6/OST1* causes a small flowering acceleration under LDs, not a delay (Zheng *et al.*, 2010). The negative role of ABA in flowering has been linked to the direct activation of *FLC* by ABA-stimulated bZIP transcriptional factor *ABSCISIC ACID-INSENSITIVE 5* (*ABI5*) and AP2/ERF domain-containing transcription factor *ABSCISIC ACID-INSENSITIVE 4* (*ABI4*) (Y. Wang *et al.*, 2013; Shu *et al.*, 2016). Such activation of *FLC* may account for the general reduction in *FT* transcript accumulation following exogenous ABA applications on leaves (Hoth *et al.*, 2002). The study of *abi1-1* plants under SDs supports this negative effect

of ABA in flowering. ABA-deficient mutants do not produce similar flowering alterations under SDs, which could depend on ABA biosynthetic mutants still producing a sufficient amount of biologically active ABA (Léon-Kloosterziel *et al.*, 1996). The early flowering of *abi1-1* plants in SDs can be completely suppressed by mutations in *SOC1*, a floral integrator activating flowering in the SAM (Searle *et al.*, 2006). Elevated levels of *SOC1* transcript in *abi1-1* mutants also suggest a negative role for ABA in *SOC1* expression, perhaps mediated by *FLC* (Fig. 6A, B). The proposed positive role of ABA-activated ABI5 on *FLC* transcriptional activation is consistent with this model (Y. Wang *et al.*, 2013).

abi1-1 plants do not present obvious flowering phenotypes under LDs despite impaired photoperiod-dependent accumulation of *FT*. We thus propose that the *abi1-1* mutants compensate for their defects in *FT* up-regulation with increased *SOC1* signalling. The late flowering phenotype of *abi1-1 soc1-1* compared with *soc1-1* under LDs is consistent with ABA playing antagonistic and spatially distinct roles in flowering, through the transcriptional activation of the florigen genes in the leaves and the repression of *SOC1* action in the shoot.

In addition to the ABA-dependent negative regulation of flowering, an ABA-independent floral repression mechanism emerged from the study of *abi1-1* plants. Under LDs, mutants of *abi1-1* exhibit a late flowering phenotype under drought stress, which is even more severe than *aba1* plants. We observed an even more pronounced delay in flowering under SDs in *abi1-1 35S::GI* plants upon drought stress compared with *35S::GI*. We interpret these results to indicate that the defects in florigen up-regulation of *abi1-1* contribute to the late flowering of *abi1-1* under drought stress. However, the levels of florigen expression in *abi1-1* were generally also low under normal watering conditions. Therefore, we hypothesize a further layer of negative regulation of flowering, which is triggered by drought stress and is probably independent of ABA (as it occurs in *abi1-1* plants). Both flowering-repressive mechanisms, the ABA-dependent and the ABA-independent mechanism, can be largely overcome under LDs, upon migration of the florigen protein in the SAM.

In conclusion, Arabidopsis plants have independent and contrasting mechanisms to modulate flowering according to water inputs; ABA stimulates GI and CO signalling to boost *FT* activation. Under drought conditions *TSF* activation is independent of *CO* and requires photoactivated GI. Simultaneously, ABA negatively regulates flowering through a pathway that requires *SOC1* (Fig. 6E), perhaps in conjunction with an ABA-independent type of regulation. Integration of these pathways in the SAM may provide plants with a flexible control of reproductive development under water stress and maximization of reproductive success.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Method used for root applications of ABA.

Figure S2. Activated ABA signalling inhibits flowering under SDs.

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

Table S2. Expression analysis of *aba elf3* mutant plants.

Table S3. Genotypes used in this study and references.

Table S4. Primers used in this study.

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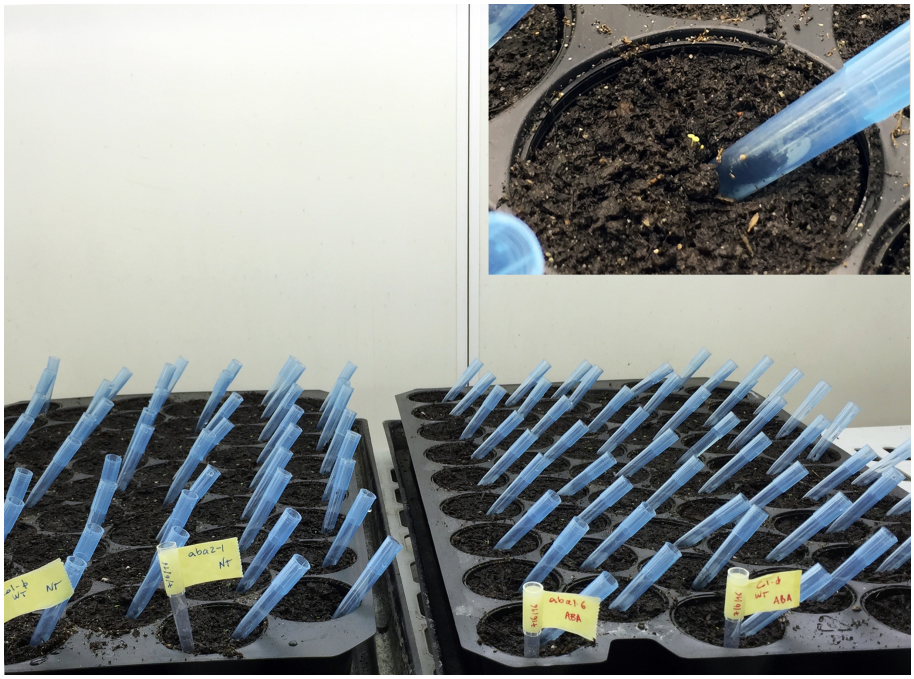

Figure S1

Method used for exogenous application of ABA. The ABA or mock solutions are applied in soil using a pipette tip as a funnel. Treatments started early in development (3 days post germination), thus before the floral transition.

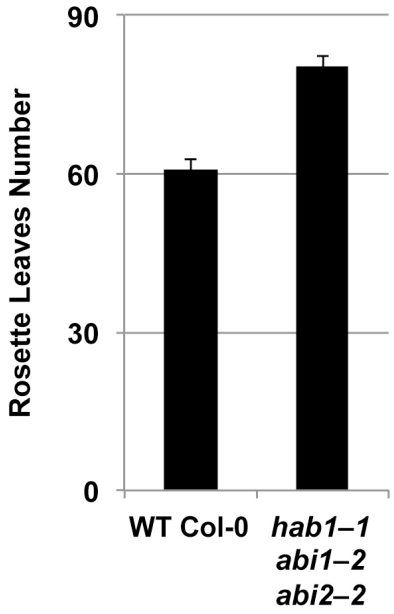

Figure S2

Activated ABA signaling inhibits flowering under SDs. Rosette leaves mean number at flowering of wild type (Col–0) and mutant hab1-1 abi1-2 abi2-2 grown under SDs. Student's t test P value ≤ 0.001 (***) compared to Wild Type.

Supplemental Table S1

Fig 1	A	Genotype	MOCK Rosette leaves	SE	range	ABA 25µM Rosette leaves	SE	range	t-test MOCK vs ABA 25µM
		WT Col–0	**10.3**	0.3	9-12	**8.6**	0.6	5-10	**P< 0.001**
		aba1–6	**17.8**	0.7	16-22	**11.9**	0.9	8-17	**P< 0.001**
		aba2-1	**16.9**	0.7	13-21	**8.8**	0.5	7-13	**P< 0.001**

One-Way ANOVA with post-HOC Tukey HSD Test

MOCK Genotype pair	p-value	inference
WT Col vs ***aba1–6***	1.01E-03	** **p<0.01**
WT Col vs ***aba2–1***	1.01E-03	** **p<0.01**
aba1–6 vs ***aba2–1***	2.77E-01	**NS**

	Normal Watering			
B Genotype	Rosette leaves	SE	range	t-test
WT Col–0	**13.9**	0.5	12–16	
aba1–6	**17.1**	0.3	16–18	**P<0.001**
ft–10	**40.9**	1.4	36–48	
ft–10 aba1–6	**53.3**	1.3	40–63	**P<0.001**
ft–10 tsf–1	**53.9**	0.6	51–55	
ft–10 tsf–1 aba1–6	**58.4**	1.4	53–64	**P=0.018**
gi–2	**61.9**	1.0	59–67	
gi–2 aba1–6	**62.0**	1.2	54–73	**P=0.945**

	Normal Watering			
C Genotype	Rosette leaves	SE	range	t-test
WT Col–0	**6.5**	0.2	5–8	
aba1–6	**7.8**	0.2	7–9	**P<0.001**
ft–10	**20.8**	0.5	19–23	
ft–10 aba1–6	**24.9**	0.5	24–28	**P<0.001**
co–10	**36.0**	0.7	31–40	
co–10 aba1–6	**36.5**	1.0	28–43	**P=0.484**

Fig 3	A	Genotype	Normal Watering Rosette leaves	SE	range	Low Watering Rosette leaves	SE	range	t-test NW vs LW
		WT Col–0	**15.9**	0.4	14–18	**11.7**	0.5	9–15	**P<0.001**
		cdf1–R cdf2–1 cdf3–1 cdf5–1	**7.8**	0.2	6–9	**5.8**	0.1	5–7	**P<0.001**
		cdf1–R cdf2–1 cdf3–1 cdf5–1 gi–100	**9.2**	0.2	8–11	**8.9**	0.2	8–10	**P=0.311**

	Normal Watering			Low Watering			
B Genotype	Rosette leaves	SE	range	Rosette leaves	SE	range	t-test NW vs LW
WT Col–0	**55.7**	0.9	51–62	**63.4**	2.4	55–75	**P=0.003**
co–10	**57.5**	1.3	51–65	**73.2**	2.8	54–85	**P<0.001**
cdf1–R cdf2–1 cdf3–1 cdf5–1	**10.5**	0.4	8–14	**10.2**	0.8	7–15	**P=0.729**

	Normal Watering			
G Genotype	Rosette leaves	SE	range	t-test
WT Col–0	**14.0**	0.0	14	
aba1-6	**22.7**	4.0	16-27	**P<0.001**
elf3-1	**4.1**	0.4	4-5	
elf3-1 aba1-6	**6.5**	1.6	4-9	**P<0.001**

Fig			Normal Watering			Low Watering			
4	A	Genotype	Rosette leaves	SE	range	Rosette leaves	SE	range	t-test NW vs LW
		WT Ler	**8.2**	0.2	7–9	**6.9**	0.1	6–8	**P<0.001**
		aba1–1	**9.4**	0.3	8–11	**9.0**	0.3	8–10	**P=0.365**
		aba1–3	**9.3**	0.2	8–11	**9.3**	0.2	8–10	**P=0.446**
		abi1–1	**8.5**	0.3	7–9	**9.5**	0.3	8–10	**P<0.001**

One-Way ANOVA with post-HOC Tukey HSD Test

Normal Watering Genotype pair	p-value	inference
WT Ler vs ***aba1–1***	1.01E-03	** **p<0.01**
WT Ler vs ***aba1–3***	3.98E-03	** **p<0.01**
WT Ler vs ***abi1–1***	8.56E-01	**NS**
aba1–1 vs ***aba1–3***	9.00E-01	**NS**
aba1–1 vs ***abi1–1***	5.83E-02	**NS**
aba1–3 vs ***abi1–1***	1.30E-01	**NS**

		Normal Watering		
B	Genotype	Rosette leaves	SE	range
	WT Ler	**37.9**	0.7	33–41
	aba1–3	**37.0**	1.2	27–42
	abi1–1	**27.0**	1.0	20–32

One-Way ANOVA with post-HOC Tukey HSD Test

Normal Watering Genotype pair	p-value	inference
WT Ler vs ***aba1–3***	7.76E-01	**NS**
WT Ler vs ***abi1–1***	1.01E-03	** **p<0.01**
aba1–3 vs ***abi1–1***	1.01E-03	** **p<0.01**

Fig			Normal Watering			Low Watering			
5	F	Genotype	Rosette leaves	SE	range	Rosette leaves	SE	range	t-test NW vs LW
		WT Ler	**35.9**	0.5	32–38	**38.4**	0.8	35–44	**P=0.016**
		abi1–1	**27.6**	0.8	23–32	**31.9**	1.3	21–36	**P=0.019**
		35S::GI	**13.6**	0.3	12–16	**11.8**	0.2	11–13	**P<0.001**
		35S::GI abi1–1	**12.6**	0.5	10–17	**17.5**	0.5	15–20	**P<0.001**

One-Way ANOVA with post-HOC Tukey HSD Test

Normal Watering Genotype pair	p-value	inference
WT Ler vs ***abi1–1***	1.01E-03	** **p<0.01**
WT Ler vs ***35S::GI***	1.01E-03	** **p<0.01**
WT Ler vs ***35S::GI abi1–1***	1.01E-03	** **p<0.01**
abi1–1 vs ***35S::GI***	1.01E-03	** **p<0.01**
abi1–1 vs ***35S::GI abi1–1***	1.01E-03	** **p<0.01**
35S::GI vs ***35S::GI abi1–1***	5.07E-01	**NS**

Fig			Normal Watering		
6	C	Genotype	Rosette leaves	SE	range
		WT Ler	**28.9**	1.4	25–37
		abi1–1	**17.0**	0.4	16–19
		soc1–1	**50.3**	1.9	42–59
		abi1–1 soc1–1	**53.7**	1.3	39–61

One-Way ANOVA with post-HOC Tukey HSD Test

Genotype pair	p-value	inference
WT Ler vs ***abi1–1***	1.01E-03	** **p<0.01**
WT Ler vs ***soc1–1***	1.01E-03	** **p<0.01**
WT Ler vs ***abi1–1 soc1–1***	1.01E-03	** **p<0.01**
abi1–1 vs ***soc1–1***	1.01E-03	** **p<0.01**
abi1–1 vs ***abi1–1 soc1–1***	1.01E-03	** **p<0.01**
soc1–1 vs ***abi1–1 soc1–1***	3.25E-01	**NS**

			Normal Watering		
6	**D**	Genotype	Rosette leaves	SE	range
		WT Ler	**8.3**	0.2	8–9
		abi1–1	**8.4**	0.2	8–9
		soc1–1	**12.5**	0.2	12–13
		abi1–1 soc1–1	**15.0**	0.2	14–16

One-Way ANOVA with post-HOC Tukey HSD Test

Genotype pair	p-value	inference
WT Ler vs ***abi1–1***	7.96E-01	**NS**
WT Ler vs ***soc1–1***	5.69E-06	***** p<0.001**
WT Ler vs ***abi1–1 soc1–1***	6.88E-15	***** p<0.001**
abi1–1 vs ***soc1–1***	7.08E-05	***** p<0.001**
abi1–1 vs ***abi1–1 soc1–1***	2.80E-14	***** p<0.001**
soc1–1 vs ***abi1–1 soc1–1***	3.62E-10	***** p<0.001**

		Normal Watering			
Fig Supp 2	Genotype	Rosette leaves	SE	range	t-test
	WT Col–0	**60.6**	2.1	49-80	
	hab1–1 abi1–2 abi2–2	**80.4**	1.9	68-101	**P<0.001**

Flowering time of mutant and transgenic plants used in this study. Mean values of vegetative leaves and standard error (SE) of plants under different watering conditions. Two tailed Student's *t* test values (*P*) and One-Way ANOVA (*P*) are shown, NS (Not Significant).

Supplemental Table S2

Fig 3			Tukey HSD	
	H	GI expression Genotype pair	p-value	inference
		WT Col–0 vs ***aba1–6***	9.00E-01	**NS**
		WT Col–0 vs ***elf3–1***	1.01E-03	**** p<0.01**
		WT Col–0 vs ***elf3–1 aba1–6***	1.01E-03	**** p<0.01**
		aba1–6 vs ***elf3–1***	1.01E-03	**** p<0.01**
		aba1–6 vs ***elf3–1 aba1–6***	1.01E-03	**** p<0.01**
		elf3–1 vs ***elf3–1 aba1–6***	9.00E-01	**NS**
			Tukey HSD	
	I	CO expression Genotype pair	p-value	inference
		WT Col–0 vs ***aba1–6***	1.29E-01	**NS**
		WT Col–0 vs ***elf3–1***	8.99E-01	**NS**
		WT Col–0 vs ***elf3–1 aba1–6***	8.96E-01	**NS**
		aba1–6 vs ***elf3–1***	3.57E-02	*** p<0.05**
		aba1–6 vs ***elf3–1 aba1–6***	3.76E-01	**NS**
		elf3–1 vs ***elf3–1 aba1–6***	5.19E-01	**NS**
			Tukey HSD	
	J	FT expression Genotype pair	p-value	inference
		WT Col–0 vs ***aba1–6***	9.00E-01	**NS**
		WT Col–0 vs ***elf3–1***	1.46E-02	*** p<0.05**
		WT Col–0 vs ***elf3–1 aba1–6***	9.00E-01	**NS**
		aba1–6 vs ***elf3–1***	6.89E-03	**** p<0.01**
		aba1–6 vs ***elf3–1 aba1–6***	9.00E-01	**NS**
		elf3–1 vs ***elf3–1 aba1–6***	1.08E-02	*** p<0.05**
			Tukey HSD	
	K	TSF expression Genotype pair	p-value	inference
		WT Col–0 vs ***aba1–6***	7.44E-02	**NS**
		WT Col–0 vs ***elf3–1***	4.35E-01	**NS**
		WT Col–0 vs ***elf3–1 aba1–6***	1.49E-01	**NS**
		aba1–6 vs ***elf3–1***	3.44E-03	**** p<0.01**
		aba1–6 vs ***elf3–1 aba1–6***	9.00E-01	**NS**
		elf3–1 vs ***elf3–1 aba1–6***	7.37E-03	**** p<0.01**

One-Way ANOVA (*P*) for the expression analysis of Fig 3H-K are shown, NS (Not Significant).

Supplemental Table S3

Allele	Reference	Background
aba1-6	(Niyogi et al., 1998)	Col-0
aba2-1	(Léon-Kloosterziel et al., 1996)	Col-0
ft-10	(Yoo et al., 2005)	Col-0
ft-10 aba1-6	This Work	Col-0
ft-10 tsf-1	(Jang et al., 2009)	Col-0
ft-10 tsf-1 aba-6	This Work	Col-0
gi-2	(Fowler et al., 1999)	Col-1
gi-2 aba1-6	This Work	Col-1
co-10	(Laubinger et al., 2006)	Col-0
co-10 aba1-6	This Work	Col-0
elf3-1	(Zagotta et al., 1992)	Col-0
elf3-1 aba1-6	This Work	Col-0
cdf1-R cdf2-1 cdf3-1 cdf5-1	(Fornara et al., 2009)	Col-0
gi-100 cdf1-R cdf2-1 cdf3-1 cdf5-1	(Fornara et al., 2009)	Col-0
aba1-1	(Koornneef et al. 1982)	L*er*
aba1-3	(Koornneef et al. 1982)	L*er*
abi1-1	(Koornneef et al., 1984)	L*er*
soc1-1	(Onouchi et al., 2000)	L*er*
abi1-1 soc1-1	This Work	L*er*
35S::GI	(Mizoguchi et al., 2005)	L*er*
abi1-1 35S::GI	This Work	L*er*
hab1-1 abi1-2 pp2ca-1	(Rubio et al., 2009)	Col-0
hab1-1 abi1-2 abi2-2	(Rubio et al., 2009)	Col-0

Genotypes used in this study

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Supplemental Table S4

Gene	Forward	Use
aba1–6	GCTCGGAGTAAAGGCGGCGA CAGGAAGTCCCCGTGACGCC	Genotyping
abi1–1	ATGGAGGAAGTATCTCCGGCG TCAGTTCAAGGGTTTGCTCTTGAG	Genotyping
CO WT	atgttgaaacaagagagtaac tcattgtgttactgttatcatctg	Genotyping
co–10	atgttgaaacaagagagtaac gcctttcagaaatggataaatagccttgcttcc	Genotyping
attb pABI1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTCTTATCCACTTTGCCCGC GGGGACCACTTTGTACAAGAAAGCTGGGTGCGATCGCCGGAGATACTTC	Cloning
ACT	CTCTCCCGCTATGTATGTCGCCA GTGAGACACACCATCACCAG	qPCR
CO	CTACAACGACAATGGTTCCATTAAC CAGGGTCAGGTTGTTGC	qPCR
FT	CTAGCAACCCTCACCTCCGAGAATA CTGCCAAGCTGTCGAAACAATATAA	qPCR
TSF	CTCGGGAATTCATCGTATTG CCCTCTGGCAGTTGAAGTAA	qPCR
SOC1	ATCGAGGAGCTGCAACAGAT GCTACTCTCTTCATCACCTCTTCC	qPCR
GI	AATTCAGCACGCGCCTATTG GTTGCTTCTGCTGCAGGAACTT	qPCR

Primers used in this study

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