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Cytokinin promotes flowering of Arabidopsis via transcriptional activation of the *FT* paralogue *TSF*

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SUMMARY

Cytokinins are involved in many aspects of plant growth and development, and physiological evidence also indicates that they have a role in floral transition. In order to integrate these phytohormones into the current knowledge of genetically defined molecular pathways to flowering, we performed exogenous treatments of adult wild type and mutant Arabidopsis plants, and analysed the expression of candidate genes. We used a hydroponic system that enables synchronous growth and flowering of Arabidopsis, and allows the precise application of chemicals to the roots for defined periods of time. We show that the application of N^6-benzylaminopurine (BAP) promotes flowering of plants grown in non-inductive short days. The response to cytokinin treatment does not require *FLOWERING LOCUS T* (*FT*), but activates its paralogue *TWIN SISTER OF FT* (*TSF*), as well as *FD*, which encodes a partner protein of TSF, and the downstream gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). Treatment of selected mutants confirmed that *TSF* and *SOC1* are necessary for the flowering response to BAP, whereas the activation cascade might partially act independently of *FD*. These experiments provide a mechanistic basis for the role of cytokinins in flowering, and demonstrate that the redundant genes *FT* and *TSF* are differently regulated by distinct floral-inducing signals.

Keywords: cytokinins, flowering, Arabidopsis, florigen, hydroponics.

INTRODUCTION

Cytokinins are important phytohormones that were first identified as factors promoting cell proliferation and shoot formation *in vitro*. These effects are now at least partly explained by the observations that cytokinins activate cell-cycle genes and interact with genetic regulators of stem cell number within the shoot apical meristem (SAM) (Riou-Khamlichi *et al.*, 1999; Rupp *et al.*, 1999; Leibfried *et al.*, 2005; Yanai *et al.*, 2005; Gordon *et al.*, 2009). Cytokinins are involved in many other aspects of plant growth and development, including vascular cambium activity, chloroplast development, response to nutrients and senescence, as well as shoot and root branching. Despite the elucidation of several pathways that regulate the transition from vegetative growth to flowering in Arabidopsis (Amasino, 2010; Fornara *et al.*, 2010), the role of cytokinins remains unclear. Repeated applications of cytokinin activated the flowering of relatively old vegetative plants, but not of younger ones (Michniewicz and Kamienska, 1965; Besnard-Wibaut, 1981; Dennis *et al.*, 1996). *In vitro*, positive (Chandler and Dean, 1994), null (Brandstatter and Kieber, 1998) or negative (Riefler *et al.*, 2006) effects were reported, suggesting that precise environmental conditions might have an effect on the response (Kinet *et al.*, 1993).

The understanding of the regulation of cytokinin synthesis, catabolism and signalling has advanced recently through the identification of genes encoding metabolic enzymes, receptors and response regulators (reviewed in Sakakibara, 2006; Hirose *et al.*, 2008; Werner and Schmülling, 2009; Kudo *et al.*, 2010; Perilli *et al.*, 2010). However, redundancy is an obstacle to using genetics to examine the biological role of cytokinins in flowering: metabolic enzymes and signalling components are encoded by multigene families, so that single mutants are similar to wild-type (WT) plants, whereas multiple mutants are impaired in growth, generating complex pleiotropic phenotypes. For example, mutants deficient in all three cytokinin receptors

isolated in Arabidopsis show reduced leaves and a stunted root. Their flowering was reported to be delayed or even suppressed, and to produce abnormal, almost sterile inflorescences (Nishimura *et al.*, 2004; Riefler *et al.*, 2006).

More physiological information was gained from plants with intermediate altered endogenous levels of cytokinins. Increased cytokinin content in the *altered meristem program 1* (*amp1*) mutant or after various chemical treatments was found to correlate with early flowering (Chaudhury *et al.*, 1993; He and Loh, 2002). In contrast, flowering was retarded in plants overexpressing CYTOKININ OXIDASE/DEHYDROGENASE (CKX), which degrades cytokinins (Werner *et al.*, 2003). These studies suggested that cytokinins stimulate flowering in Arabidopsis. Furthermore, an increased level of cytokinins was found in the SAM of Arabidopsis plants induced to flower by a single long day (LD) (Corbesier *et al.*, 2003). This increase was preceded by the elevation of cytokinin content in leaf extracts and leaf exudate, suggesting that cytokinins might be involved in flowering as systemic signals, as discussed elsewhere (Bernier and Périlleux, 2005).

Cytokinins remain to be integrated into the current knowledge of genetically defined molecular pathways to flowering (reviewed in Davis, 2009). In response to LDs, the mobile protein FLOWERING LOCUS T (FT) exerts a prominent role (Turck *et al.*, 2008). Under these conditions, the *FT* gene is activated in the leaf phloem by CONSTANS (CO), a B-box zinc-finger transcription factor (Suárez-López *et al.*, 2001). The FT protein is thereafter transported to the SAM, where it promotes flowering by reprogramming transcription (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007). FT forms a complex with the transcription factor FD that activates *APETALA 1* (*AP1*), a MADS box gene that confers – together with *LEAFY* (*LFY*) – floral identity on primordia (Abe *et al.*, 2005; Wigge *et al.*, 2005). FT and FD are also required for the activation of another MADS box gene, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), which is the first gene known to be activated in the SAM after exposure to LDs (Borner *et al.*, 2000; Samach *et al.*, 2000; Searle *et al.*, 2006; Jang *et al.*, 2009), and activates *LFY* (Lee *et al.*, 2008; Liu *et al.*, 2008). The *FT* paralogue *TWIN SISTER OF FT* (*TSF*) acts as a floral stimulus redundantly with *FT*, sharing activation by CO and binding of the protein to FD (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005; Jang *et al.*, 2009).

The putative targets of cytokinins during the floral transition in Arabidopsis are investigated in this study. We performed cytokinin treatment on adult vegetative plants, and followed the expression of candidate flowering-time genes at different positions within the genetic cascade of floral transition. To ensure reproducibility of the experiments, a hydroponic system was used that enables synchronous growth and flowering of Arabidopsis (Tocquin *et al.*, 2003), as well as the precise application of chemicals to the roots for defined periods of time. We show that cytokinin treatment promotes flowering, and that this occurs independently of *FT*, through transcriptional activation of *TSF*. These experiments provide a mechanistic basis for the role of cytokinins in flowering.

RESULTS

Plants of Arabidopsis WT Columbia were grown in hydroponics in 8-hour short days (SDs). In the experimental conditions used, flowering under SDs was very late and asynchronous; floral buds were macroscopically visible in 50% of the population after about 120 days (17 weeks). When plants were 7-weeks old, they were exposed to a single 22-h LD, kept under SDs as a control or were kept under SDs and treated once with N^6-benzylaminopurine (BAP). The start of the light period on the experimental day is referred to as 'hour 0'. BAP was applied during the night from hour 8 to hour 16, and was supplied to the roots via the hydroponic solution to obtain final concentrations of 0.5, 5 or 50 μM BAP. This mode of application was selected because it allows for the precise control of dose and timing. Furthermore, cytokinins have previously been suggested to act as root-to-shoot signals at floral transition (Kinet *et al.*, 1993; Havelange *et al.*, 2000). At hour 16, the BAP treatment was terminated by changing to fresh, hormone-free, hydroponic solution. Plants were observed 2 weeks later to evaluate their flowering response. They were classified as floral when floral buds were visible under the dissecting microscope. All plants exposed to an LD formed floral buds within 2 weeks, whereas plants maintained under SDs did not (Figure 1a); this result is consistent with previous reports that one LD is sufficient to induce the flowering of Arabidopsis plants at this age (Corbesier *et al.*, 1996; King *et al.*, 2008), including plants grown in hydroponics (Tocquin *et al.*, 2003). Most interestingly, the application of BAP to the roots stimulated flowering under SDs: this effect increased with the final concentration of BAP, and almost 100% of the plants initiated floral buds within 2 weeks when treated with 50 μM BAP. This treatment was therefore selected as 'standard' for further investigations. In total, 17 experiments were conducted: 100% of the plants flowered in response to BAP in 12 experiments, whereas at least 75% flowered in the remaining five. The BAP treatment was also tested on younger, 5-week-old plants, but the flowering response was less reproducible, so that between 45 and 100% of plants flowered in five independent experiments.

The florigenic effect of BAP is rapid

Although the BAP treatment was transient, at the time of dissection the treated plants showed a reduced rosette diameter compared with the control SD plants, and the youngest leaves were smaller and serrate (Figure 1b). They were also greener, as confirmed by their higher chlorophyll

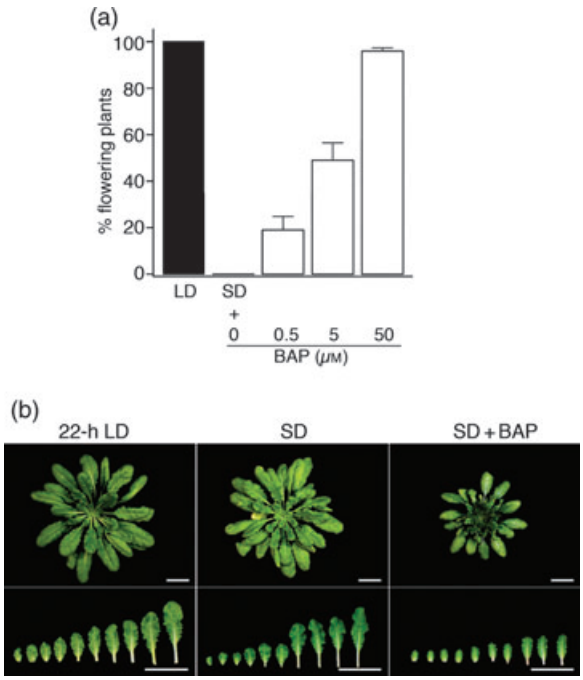

Figure 1. Exogenous cytokinin promotes flowering under short days (SDs).
(a) Flowering response of 7-week-old vegetative plants grown in 8-h SDs and exposed to one 22-hour long day (LD) (black bars) or to N^6-benzylaminopurine (BAP) treatment (white bars). BAP was added to the hydroponic solution for 8 h to achieve the final concentrations as indicated. The results are percentages (±SEs) of plants initiating floral buds within 2 weeks after treatment. Data were calculated from three independent experiments, each involving 15 plants.
(b) Representative photographs of the plants 2 weeks after treatments. Leaves shown are the 10 youngest ones longer than 5 mm. Scale bars: 2 cm.

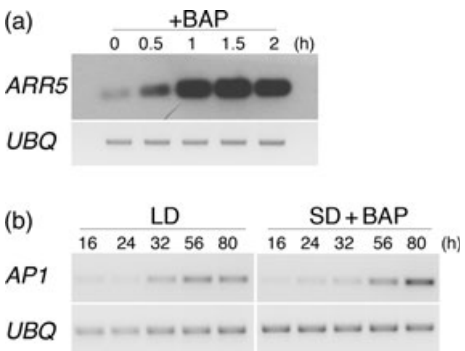

Figure 2. The effect of N^6-benzylaminopurine (BAP) is rapid.
(a) Transcript level of *ARR5* in shoot apices. Time is expressed as hours (h) after the start of standard BAP treatment.
(b) Transcript level of *AP1* in shoot apices of plants exposed to one 22-h long day (LD) or maintained under 8-hour SDs and exposed to standard BAP treatment. Time is expressed as hours (h) after the start of the experimental day.

content (Table S1). By contrast, no effect of BAP on root growth was observed, as evaluated by root dry weight.

In order to estimate the delay between BAP treatment and floral transition, the expression of the *Arabidopsis RESPONSE REGULATOR 5* (*ARR5*) gene, which is transcriptionally upregulated within minutes of cytokinin application, was measured (Brandstatter and Kieber, 1998). Similarly, *AP1* mRNA, which is a marker for floral meristems, was tested (Hempel *et al.*, 1997). Upregulation of *ARR5* mRNA was detected in shoot apices just half an hour after the addition of BAP to the hydroponic solution (Figure 2a). Upregulation of *AP1* mRNA occurred approximately 56 h after the start of the experimental day (Figure 2b), indicating that floral meristems were initiated about 2 days after BAP treatment, which is approximately the same kinetics as after exposure to the 22-h LD. Upregulation of *AP1* did not occur under SDs within the investigated period (not shown).

Critical flowering-time genes are induced by BAP

The expression of flowering-time genes was analysed in response to BAP treatment. Previous studies indicated a link between cytokinins and the induction of flowering by one LD (Corbesier *et al.*, 2003), so we focused on genes of the photoperiodic pathway: *CO*, *FT*, *TSF*, *SOC1* and *FD*. Leaf samples were analysed and complementary *in situ* hybridizations were performed for *SOC1* and *FD* at the SAM, where these genes are mostly expressed (Borner *et al.*, 2000; Samach *et al.*, 2000; Abe *et al.*, 2005; Wigge *et al.*, 2005). *CO*, *FT* and *TSF* transcripts were analysed by RT-PCR because their abundance is too low to be detected by other methods (Takada and Goto, 2003; An *et al.*, 2004; Yamaguchi *et al.*, 2005).

Consistent with previous reports (Suárez-López *et al.*, 2001; Corbesier *et al.*, 2007), *CO* mRNA showed a peak during the dark period under SDs, which extended into the day under an inductive LD (Figure 3a). This extension of the *CO* mRNA peak was not observed after BAP treatment under SDs.

FT and *TSF* mRNAs were barely or not expressed under SDs, but showed an immediate upregulation upon day-length extension, giving an evening peak during the LD (Figure 3a). BAP treatment had no effect on *FT* mRNA under SDs, but *TSF* mRNA levels were increased in the leaves. This activation was detectable at hour 32, and was clearly apparent at hours 56 and 80. Both the absence of induction of *FT* mRNA by BAP and the activation of *TSF* were confirmed in independent experiments by qRT-PCR (Figure 3b).

SOC1 expression was detected in leaves and shoot apices (Figures 3a and 4). In leaves (Figure 3a), a peak of *SOC1* transcript abundance was detected at hour 8 under SDs, as described by Blázquez *et al.* (2002). At hours 16 and 24, *SOC1* transcripts had decreased to similar levels under all three conditions, and hence no effect of the LD or the BAP treatment was detected. Circadian regulation of *SOC1* could explain the similar levels detected at hours 8, 32, 56 and 80 (Blázquez *et al.*, 2002). By contrast, in shoot apices, *SOC1*

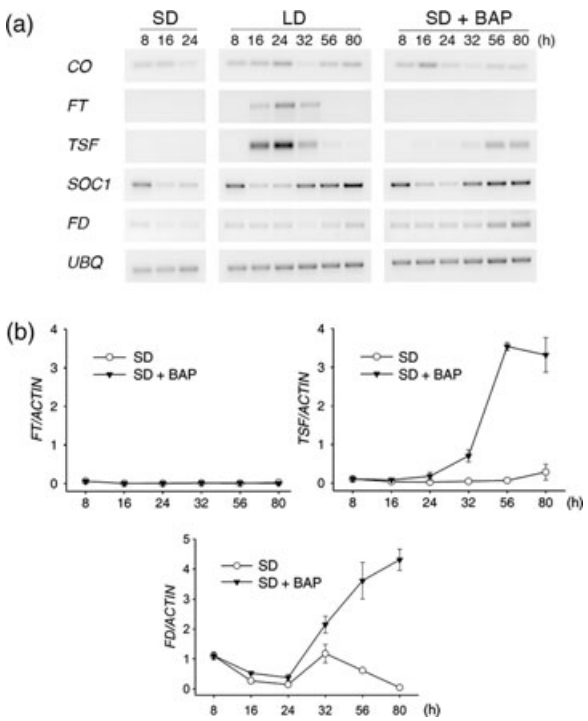

Figure 3. Flowering-time genes are activated by N^6-benzylaminopurine (BAP) in the leaves.
(a) Transcript levels of *CO*, *FT*, *TSF*, *SOC1* and *FD* as evaluated by semiquantitative RT-PCR. Plants were kept under control 8-h short days (SDs), exposed to one 22-h long day (LD) or were kept under SDs and exposed to standard BAP treatment. Time is expressed as hours (h) after the start of the experimental day.
(b) Quantification of *FT*, *TSF* and *FD* transcripts by qRT-PCR in an independent experiment.

mRNA abundance was strongly upregulated in response to the LD, as reported previously (Borner *et al.*, 2000), and this also occurred after BAP treatment (Figure 4). *In situ* hybridization revealed upregulation of *SOC1* mRNA from hour 24, which is 8 h after the end of the BAP treatment.

Expression of *FD* mRNA was detected in leaves and shoot apices, even under SDs (Figures 3a and 4). The transcript level increased in the shoot apices in response to the inductive LD (Figure 4), as reported previously (Wigge *et al.*, 2005; Searle *et al.*, 2006), but we did not detect any change in the leaves (Figure 3a). By contrast, after BAP treatment, the upregulation of *FD* was detected in the SAM from hour 32 (Figure 4), and was also detected in the leaves (Figure 3a). The induction of *FD* mRNA by BAP in the leaves was confirmed in independent experiments by qRT-PCR (Figure 3b).

TSF and SOC1 are required for a flowering response to cytokinin

The standard BAP treatment was tested on *ft-10*, *tsf-1*, *fd-5* and *soc1-2* single mutants, and on *ft-10 tsf-1* and *tsf-1 soc1-2* double mutants. The *ft-10* null mutants formed floral buds

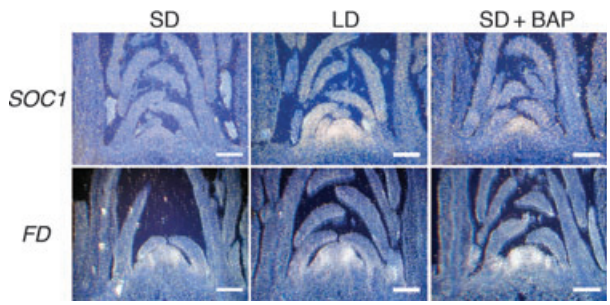

Figure 4. *In situ* hybridization of *SOC1* and *FD* probes on shoot apices harvested 32 h after the start of the experiment. Plants were kept under control 8-h short days (SDs), were exposed to one 22-h long day (LD) or were kept under SDs and exposed to standard treatment with N^6-benzylaminopurine (BAP). Scale bars: 100 μm.

Table 1 Flowering response of 7-week-old wild-type (WT) plants and flowering-time mutants to standard treatment with N^6-benzylaminopurine (BAP)

	% Flowering plants	
Genotype	SD	SD + BAP
Col WT	0 ± 0	88.5 ± 4.4
ft-10	0 ± 0	85.2 ± 4.8
tsf-1	0 ± 0	0 ± 0
soc1-2	0 ± 0	0 ± 0
fd-5	0 ± 0	35.4 ± 6.9
tsf-1 soc1-2	0 ± 0	0 ± 0
ft-10 tsf-1	0 ± 0	0 ± 0

All plants were grown under 8-h short days (SDs). Results are percentages (±SEs) of plants having initiated floral buds 2 weeks after treatment. Data were calculated from three independent experiments, each involving 18 plants.

within 2 weeks of BAP treatment (Table 1), suggesting that *FT* is not required for the florigenic effect of the treatment. By contrast, *tsf-1* and *soc1-2* did not respond to BAP, indicating that *TSF* and *SOC1* are required in the molecular pathway that initiates flowering in response to BAP. These observations were confirmed by SEM examination of apical meristems (Figure 5). Floral buds were clearly initiated after

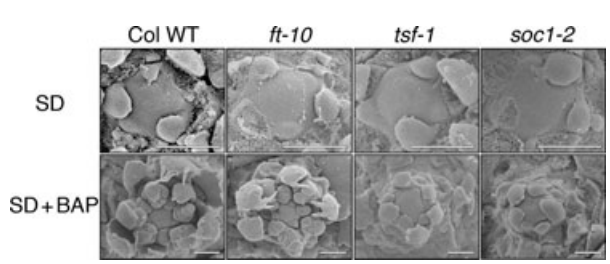

Figure 5. Scanning electron micrographs of wild-type (WT), *ft-10*, *tsf-1* and *soc1-2* shoot apices 2 weeks after standard treatment with N^6-benzylaminopurine (BAP; lower row), as compared with untreated plants (upper row). Scale bars: 100 μm.

BAP treatment in the *ft-10* mutant, as in the WT, whereas the SAM of *tsf-1* and *soc1-2* mutants continued growing vegetatively. Also consistent with the suppression of the flowering response to BAP by a loss of *TSF* or *SOC1* function, the double mutants *ft-10 tsf-1* and *tsf-1 soc1-2* remained vegetative after the treatment (Table 1). By contrast, *fd-5* mutant showed a reduced response: 35% of the individuals formed visible floral buds within 2 weeks after the BAP treatment. A similar proportion of flowering plants was obtained with the *fd-3* mutant, in two independent experiments (not shown).

DISCUSSION

Flowering is a key step in plant development, and recent research on Arabidopsis has revealed complex networks of genetic regulatory pathways (reviewed in Amasino, 2010; Fornara *et al.*, 2010). Much information has been gained towards an understanding of the molecular cascades whereby flowering is controlled by environmental cues, especially photoperiod and vernalization, whereas endogenous flowering signals have been more difficult to investigate. Plant hormones regulate multiple aspects of growth and development, so that it is hard to discriminate the direct and indirect effects that mutations in their signalling components might have on late phenotypic traits, such as flowering. A similar difficulty is experienced after prolonged exposures to exogenously applied hormones. Gibberellins (GAs) are the class of hormones in which involvement in the transition to flowering in Arabidopsis is best documented. The existence of a GA pathway to flowering was inferred from the late flowering of GA-deficient single mutants (Wilson *et al.*, 1992), the identification of molecular targets among flowering-time genes, such as *SOC1* (Borner *et al.*, 2000) and *LFY* (Blázquez *et al.*, 1998), and the careful analysis of GA synthesis and transport during the transition to flowering (Eriksson *et al.*, 2006). These studies showed that GAs are required for flowering under SDs, whereas their contribution is less important under LDs (Hisamatsu and King, 2008). By contrast, very little is known about other hormones, although classical studies have implicated several of them in the floral transition. There is a need to integrate physiological approaches with genetics to build a comprehensive model for hormone activity during the floral transition (Davis, 2009). We have studied the effects of cytokinins.

Only in the past decade have the molecular bases of cytokinin signalling been uncovered, and important gene redundancy has been found among cytokinin signalling components (Hirose *et al.*, 2008). To avoid the pleiotropic effects that multiple mutations or long hormonal treatments might induce, we addressed the question of the cytokinin function in the flowering of Arabidopsis by using an experimental system that allowed the synchronous growth of the plants, the control of floral transition (Tocquin *et al.*, 2003) and the transient application of chemicals. We observed and reproduced in a large number of independent experiments that an 8-h application of 50 μM BAP to the roots of 7-week-old vegetative plants of Arabidopsis grown under SDs, in hydroponics, strongly promoted flowering in the absence of all known inducing factors: LD, vernalization or exogenous GA. This effect was not pleiotropic because the *AP1* mRNA was upregulated in shoot apices approximately 2 days after the end of the BAP treatment (Figure 2b), indicating that floral transition is a fast response to BAP treatment, and not an indirect consequence of altered growth. The high efficiency of BAP in promoting flowering in our experimental system contrasts with the variability that emerges from previous studies (see Introduction). Many factors, including the mode of application (site, time and dose), the endogenous status of the plants (possibly their cytokinin content) and the environmental conditions might account for these discrepancies (Kinet *et al.*, 1993). Cytokinins are well documented to interact with environmental signalling such as nutrient sensing (Argueso *et al.*, 2009), and hence the importance of controlling all parameters – including substrate – might have been underestimated so far. Supporting this assumption, Miyawaki *et al.* (2006) noticed that multiple mutants in cytokinin biosynthesis genes were delayed in flowering on vermiculite but not on nutrient agar.

Traditionally it was thought that cytokinins act as long-distance signals of root origin, because they were found in the xylem sap of several species (reviewed in Hirose *et al.*, 2008; Kudo *et al.*, 2010). This view was simplified, as demonstrated recently by studies on the spatial expression patterns of cytokinin signalling components. Nevertheless, we applied BAP in the hydroponic solution and observed the very rapid activation of the cytokinin-inducible gene *ARR5* in the shoot apex during treatment (Figure 2a), indicating fast uptake of BAP by the roots and immediate transport upwards. Hence, BAP might promote flowering by direct action in the SAM. However, classical experiments suggested a more complex cytokinin route to flowering. In the LD plant *Sinapis alba*, the analysis of phloem and xylem sap during the induction of flowering by a single LD involved cytokinins in a shoot-to-root-to-shoot signalling loop (Havelange *et al.*, 2000). An increased export of cytokinins from the roots was detected during a single inductive LD, and was shown to be triggered by a shoot-derived signal, probably sucrose. At the same time, the cytokinin content increased in leaf exudates and in the SAM (reviewed in Bernier and Périlleux, 2005). Similar changes were reported in Arabidopsis, but, because of technical limitations, only shoots were analysed. An increased export of cytokinins out of the leaves was observed upon photoperiodic induction of flowering, as well as SAM enrichment in active cytokinins (Corbesier *et al.*, 2003). In both the Arabidopsis and *Sinapis* cases, translocation of the flowering signals during the inductive

LD had been evaluated by defoliation experiments, and the timing of the cytokinin export towards the SAM was consistent with the idea that these hormones could be part of the systemic flowering signals. We therefore analysed the expression of candidate flowering-time genes after BAP treatment in leaves and shoot apices.

Most interestingly, we observed that BAP treatment activated the transcription of *TSF* but not *FT* in the leaves (Figure 3). The functional relevance of these expression patterns was confirmed by mutant analyses (Table 1): the loss of *TSF* function suppressed the flowering response to BAP, whereas the loss of *FT* had no effect (Figure 5; Table 1). These results clearly demonstrate that a cytokinin route to flowering in Arabidopsis bypasses *FT* but requires its paralogue *TSF*.

The BAP treatment also induced upregulation of *SOC1*, at least in the SAM (Figure 4). This observation is consistent with a report on *Sinapis*, where exogenous cytokinin applied on the shoot apex induced the *SOC1* orthologue *SaMADSA* (Bonhomme *et al.*, 2000). It is worth noting that, in *Sinapis*, the cytokinin treatment triggered other changes that are observed in the SAM during the transition to flowering – such as mitotic activation (Bernier *et al.*, 1977) and secondary plasmodesmata formation (Ormenese *et al.*, 2006) – but was not sufficient on its own to reach floral bud initiation. We show here that, in Arabidopsis, the standard BAP treatment enables the complete floral transition of the SAM (Figure 5), and that *SOC1* is absolutely required in the molecular pathway: the florigenic effect of BAP treatment is indeed suppressed in the *soc1-2* mutant, as in *tsf-1* (Table 1). Genetic molecular studies showed that *SOC1* acts downstream of *FT* and *TSF* in LDs (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005; Yoo *et al.*, 2005; Jang *et al.*, 2009). Therefore, *SOC1* is presumably required for *TSF* to promote flowering after BAP treatment in SD. We cannot exclude, however, that upregulation of *SOC1* by BAP might proceed independently of *TSF*, and that both are necessary for early flowering. These results add to the role of *SOC1* as an integrator of multiple signals mediating the environmental, age-dependent and hormonal regulation of flowering (Lee and Lee, 2010).

Whether the florigenic effect of BAP passes through *FD* is not clear: although the *FD* mRNA level was increased in response to BAP treatment (Figures 3a and 4), the loss of *FD* function lowered, but did not suppress, the flowering response to BAP (Table 1). This suggests that *FD* function might be shared by other gene(s), such as *FD PARALOGUE* (*FDP*) (Abe *et al.*, 2005; Wigge *et al.*, 2005). Activation of *TSF* and *FD* by BAP (Figures 3 and 4), and direct interaction between TSF and FD or FDP proteins (Jang *et al.*, 2009), suggest that a TSF/FD(P) complex is involved in promoting flowering after BAP treatment. We cannot exclude that the TSF/FD complex may function in the leaves, as both partners were upregulated at about the same time in the leaves by BAP (Figure 3). This was quite unexpected because *FD* is mostly expressed in shoot and root apices in Arabidopsis (Abe *et al.*, 2005; Wigge *et al.*, 2005). However, *FD* was found to be required for *FT* to increase gene expression in leaves (Teper-Bamnolker and Samach, 2005), and, in tomato, the *FD* homologue *SPGB* is expressed in both the leaves and shoot apices (Lifschitz *et al.*, 2006). Alternatively, the fact that the TSF protein was found in phloem sap (Giavalisco *et al.*, 2006) suggests that it might be a systemic signal. This suggests a model whereby BAP activates flowering by inducing the expression of TSF, which moves to the meristem and, through interaction with FD or FDP, brings about activation of *SOC1* and *AP1* transcription.

Taken together, the results described here highlight a flowering pathway where the relative contributions of *FT* and *TSF* are reversed, as compared with the effect of exposure to LDs. In LDs, although both *FT* and *TSF* are transcribed, only the loss of *FT* function strongly delays flowering, whereas abolishing the activity of *TSF* alone has a weak effect (Jang *et al.*, 2009). Here, we show that BAP can trigger flowering in SDs and activates the transcription of *TSF*, whereas *FT* activity is not necessary. Activation of *TSF* by cytokinins might occur under LDs, where endogenous cytokinin levels are increased, at least in the leaves and SAM (Corbesier *et al.*, 2003), but also in any other conditions where cytokinins act as a signalling cue to relay information from the environment. Such a model suggests that different transcriptional control of *FT* and *TSF* could be the basis of flowering responses to different environmental or internal signals.

EXPERIMENTAL PROCEDURES

Plant growth and material

The hydroponic set-up was as described by Tocquin *et al.* (2003): the nutrient solution was previously referred to as '1N-supply' (Tocquin *et al.*, 2006; Table S2). The mutant experiments were performed with the Araponics growing device (http://araponics.com). Light was provided by cool-white fluorescent tubes at 50 μmol m^{-2} s^{-1}. The temperature was 20°C/18°C (day/night), and the relative humidity was 70%.

Arabidopsis thaliana ecotype Columbia (Col) was used throughout. *ft-10* is a GABI-Kat T-DNA insertion line named 290E08 (http://www.gabi-kat.de). *tsf-1*, *soc1-2*, *fd-5* and *fd-3* are T-DNA lines from the SALK collection: *tsf-1* is line SALK_087522 (previously described in Michaels *et al.*, 2005); *soc1-2* is line SALK_138131 (previously described as *agl20* by Lee *et al.*, 2000); *fd-3* is line SALK_054421 (previously described in Abe *et al.*, 2005); *fd-5* is line SALK_150991. The *ft-10 tsf-1* (previously described in Jang *et al.*, 2009) and *soc1-2 tsf-1* double mutants were generated by crossing the single mutants. Plants homozygous for both mutations were obtained, and the alleles were genotyped by PCR.

RT-PCR

Shoot apices (2 mm) and remaining rosette leaves were harvested from 15 plants per batch. RNA was extracted in TRIzol (Invitrogen, http://www.invitrogen.com). For semiquantitative RT-PCR, cDNA was synthesised as previously described (D'Aloia *et al.*, 2009) from

1 μg RNA and a 1/15 volume was used for PCR. Gels of PCR products were stained with ethidium bromide (*AP1*, *CO*, *FD*, *FT*, *TSF*, *SOC1* and *UBQ10*) or were blotted (*ARR5* and *UBQ10*). Hybridization of Southern blots was performed with DIG-labelled cDNA probes (full-length cDNA for *ARR5* and a fragment from nt +988 to +1515 for *UBQ10*, both cloned in pCR2.1 plasmid and amplified with M13 primers) using the Dig High Prime DNA Labeling and Detection Starter Kit, as recommended by the manufacturer (Roche, http://www.roche.com).

For qRT-PCR, cDNA was synthesised from 3 μg RNA and a 1/50 volume was used for PCR. PCR reactions were performed in triplicate using SYBR-Green I and the IQ5 cycler (Bio-Rad, http://www.bio-rad.com). *ACT2* was used for normalization. Primers are listed in Table S3.

In situ hybridization

In situ hybridization was performed as previously described in Thouet *et al.* (2008). [^{35}S]UTP-RNA labelled probes were produced from cDNA fragments of *SOC1* (from nt +166 to +848) and *FD* (from nt +83 to +587) cloned in Bluescript and pGEM-T vectors, respectively. *SOC1* antisense and sense probes were synthesized with T3 and T7 RNA polymerases, respectively, according to the manufacturer's instructions (Promega, http://www.promega.com), after plasmid linearization with *Hind*III and *Bam*HI. *FD* antisense and sense probes were synthesized with T7 and Sp6 RNA polymerases, respectively, after cDNA amplification by PCR with M13 primers.

Scanning electron microscopy

Shoot apices were harvested 2 weeks after BAP treatment and fixed overnight at 4°C in 2% glutaraldehyde : 0.1 M sodium phosphate buffer (pH 7.2). Samples were thereafter rinsed in buffer and post-fixed for 1 h in 1% OsO_4 at 4°C. After dehydration through an ethanol series, samples were critical-point dried with CO_2 and sputter-coated with gold-palladium. The specimens were examined with a JEOL scanning electron microscope (http://www.jeol.com) at 19 kV.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Morphological effects of standard BAP treatment.

Table S2. Composition of the hydroponic solution.

Table S3. Primers and conditions used for RT-PCR.

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