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RESEARCH PAPER

HAHB10, a sunflower HD-Zip II transcription factor, participates in the induction of flowering and in the control of phytohormone-mediated responses to biotic stress

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Abstract

The transcription factor HAHB10 belongs to the sunflower (*Helianthus annuus*) HD-Zip II subfamily and it has been previously associated with the induction of flowering. In this study it is shown that *HAHB10* is expressed in sunflower leaves throughout the vegetative stage and in stamens during the reproductive stage. In short-day inductive conditions the expression of this gene is induced in shoot apexes together with the expression of the flowering genes *HAFT* and *HAAP1*. Transgenic *Arabidopsis* plants expressing *HAHB10* cDNA under regulation either by its own promoter or by cauliflower mosaic virus (CaMV) 35S exhibited an early flowering phenotype. This phenotype was completely reverted in a non-inductive light regime, indicating a photoperiod-dependent action for this transcription factor. Gene expression profiling of *Arabidopsis* plants constitutively expressing *HAHB10* indicated that specific flowering transition genes such as *FT*, *FUL*, and *SEP3* were induced several fold, whereas genes related to biotic stress responses, such as *PR1*, *PR2*, *ICS1*, *AOC1*, *EDS5*, and *PDF1-2a*, were repressed. The expression of *HAHB10* and of the flowering genes *HASEP3* and *HAFT* was up-regulated by both salicylic acid (SA) treatment and infection with a virulent strain of *Pseudomonas syringae.* Basal SA and jasmonic acid (JA) levels in *Arabidopsis* plants ectopically expressing *HAHB10* were similar to those of control plants; however, SA levels differentially increased in the transgenic plants after wounding and infection with *P. syringae* while JA levels differentially decreased. Taken together, the results indicated that *HAHB10* participates in two different processes in plants: the transition from the vegetative to the flowering stage via the induction of specific flowering transition genes and the accumulation of phytohormones upon biotic stresses.

Key words: *FT*, *HAHB10*, HD-Zip, plant defence mechanisms, salicylic acid, *SEP3*, sunflower transcription factor.

Introduction

The transition from the vegetative to the reproductive stage occurs via complex mechanisms involving genes participating in different signal transduction pathways (Levy and Dean, 1998). The timing of this process is governed by several external factors such as light quality, photoperiod, and vernalization (Levy and Dean, 1998). Additionally, biotic and abiotic stresses can accelerate the entrance into the reproductive stage. For example, salicylic acid (SA), a critical hormone involved in the response to pathogens (Wildermuth, 2001; Loake and Grant, 2007; Park *et al.*,

Abbreviations: ABA, abscisic acid; *AP1*, *APETALLA1*; *CO*, *CONSTANS*; ET, ethylene; *FT*, flowering time; *FUL*, *FRUITFULL*; *HAHB10*, *Helianthus annuus* homeobox 10; JA, jasmonic acid; LD, long day regime; SA, salicylic acid; SD, short day regime; *SEP3*, *SEPALLATA3*.

2007), has been described as an inducer of flowering in several plant species (Cleland and Ajami 1974; Khurana and Cleland, 1992). Martinez *et al.* (2004) analysed the transcript levels of several genes participating in flowering in SA-deficient plants and found that the *FLOWERING TIME* (*FT*) gene displays lower expression levels in these plants than in wild-type plants. Concomitantly, stress conditions increasing the endogenous SA content activate the expression of *FT* (Martínez *et al.*, 2004). The ectopic expression of the *Arabidopsis* and tomato (*Solanum lycopersicum*) *FT* genes causes both early flowering (Kardailsky *et al.*, 1999; Carmel-Goren *et al.*, 2003) and enhanced levels of *FRUITFULL* (*FUL*) and *SEPALLATA3* (*SEP3*) gene expression (Teper-Bamnolker and Samach, 2005).

SA also participates as a signal molecule during infection with bacterial pathogens to control the expression of specific defence genes (Després *et al.*, 2000; Spoel *et al.*, 2003; Weigel *et al.*, 2005). In contrast to SA, jasmonic acid (JA) levels usually decrease during bacterial infection, but they increase after wounding or attack by herbivores or necrotrophic pathogens (Balbi and Devoto, 2008). Although both hormones trigger specific healing and defence responses in plants, a very delicate balance between the levels of these two hormones is required to tailor these responses (Anderson *et al.*, 2004). Moreover, SA and JA also interact with ethylene and abscisic acid (ABA) during defence responses to act either synergistically, additively, or antagonistically depending on the stimuli (Xu *et al.*, 1994; O'Donnell *et al.*, 1996; Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003; Anderson *et al.*, 2004).

In a previous study, it was reported that *HAHB10*, a sunflower HD-Zip transcription factor belonging to subfamily II, was able to accelerate flowering and thereby shortens the plant's life cycle when ectopically expressed in *Arabidopsis* plants (Rueda *et al.*, 2005). Transcription factors from the HD-Zip family are characterized by the association of an HD with a LZ domain, an association unique to plants (Chan *et al.*, 1998). These proteins are classified in four subfamilies (I–IV) according to sequence conservation, gene structures, and functions, among other features (Chan *et al.*, 1998; Ariel *et al.*, 2007). Members of the subfamily II (like HAHB10) bind *in vitro* to the pseudopalindromic sequence CAAT(C/G)ATTG and those that have been characterized thus far are involved in light signalling pathways. For example, *Arabidopsis ATHB2*/*HAT4* induces early flowering when ectopically expressed and it has been described as a master switch in the shade avoidance response (Schena *et al.*, 1993; Steindler *et al.*, 1999; Ciarbelli *et al.*, 2008; Sorin *et al.*, 2009). Several HD-Zip transcription factors from subfamily I, first assigned as regulators of abiotic stress responses, have been shown also to participate in biotic stress responses. For example, the sunflower *HAHB4* confers drought tolerance and enhanced defence responses against insect attack when ectopically expressed in *Arabidopsis* (Manavella *et al.*, 2008), the tomato *H52* participates in the regulation of pathogen resistance and cell death (Mayda *et al.*, 1999), and the *Nicotiana benthaniana NbHB1* is a JA-dependent positive regulator of pathogen-induced plant cell death (Yoon *et al.*, 2009).

In this study, it was demonstrated that *HAHB10* induces flowering by affecting the expression of specific genes operating during the vegetative to flowering transition. In addition to the up-regulation of flowering genes in these plants, several genes involved in biotic stress responses were repressed. Levels of SA, JA and ethylene were not changed in *Arabidopsis* plants expressing *HAHB10* in control conditions but were significantly affected after bacterial infection and wounding, and these plants were affected in their response to a compatible interaction with *Pseudomonas syringae*.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) and *Helianthus annuus* L. (sunflower CF33 cv. from Advanta) were grown in a growth chamber at 22–24 °C or 26–28 °C, respectively, under long-day or short-day photoperiods (LD or SD) as indicated in the figures (16 h or 8 h of illumination by a mixture of cool-white and GroLux fluorescent lamps). The light intensity in the culture chamber was ~150 μE m^{-2} s^{-1}.

Arabidopsis plants were grown in Petri dishes containing 0.8% agar–Murashige and Skoog (MS) medium or in soil pots (8×7 cm) depending on the experiment and during the periods indicated in the figures.

Sunflowers were grown in 27×30 cm soil pots until different developmental stages as defined by Shneiter (1981). In order to test inductive conditions, V10 plants were placed under a 8 h illumination regime during 96 h while their controls remained in LD conditions.

Constructs

For isolation of the *HAHB10* promoter, a sunflower bacterial artificial chromosome (BAC) library (BAC Library HA_HBa, CUGI-Clemson University Genome Institute) was screened with a ^{32}P-labelled probe corresponding to the 5′-non-coding region of the *HAHB10* cDNA plus the first 241 nucleotides of the coding region, which does not include the HD-Zip domain (*Eco*RI/*Spe*I fragment). One of the isolated positive clones was digested with *Eco*RI and *Hin*dIII, and analysed in a Southern blot using the same probe. A 3025 bp hybridized fragment was subcloned in the pBluescript SK vector and sequenced (Macrogen-Korea). A 1399 bp DNA fragment (accession no. GQ470994) located upstream of the transcription initiation site was amplified by PCR using the oligonucleotides H10-1R (5′-CCGGGATCCCCATCTGAATAAAAAATGTGT-3′) and H10-8F (5′- CGCAAGCTTCTTGGTACCGATACCCAGAAC-3′) bearing the *Bam*HI and *Hin*dIII sites, restricted with these enzymes, and cloned in the pBI 101.3 binary vector directing expression of the *GUS* (β-glucuronidase) reporter gene.

The construct *35S:HAHB10* in the pBI121 vector was previously described (Rueda *et al.*, 2005).

PromHAHB10:HAHB10 was obtained by replacing the 35S cauliflower mosaic virus (CaMV) promoter (by *Bam*HI/*Hin*dIII restriction) by the *HAHB10* promoter fragment (1399 bp) in the above-mentioned *35S:HAHB10* construct.

Escherichia coli DH5α cells were transformed with each construct and, once a positive clone was identified, it was used to transform *Agrobacterium tumefaciens* cells (LBA4404) (Höfgen and Willmitzer, 1988).

Transformation and identification of transformed plants

Stable transformation of *Arabidopsis* plants was carried out by the floral dip procedure (Clough and Bent, 1998). At least three independent homozygous lines of each transgenic genotype were used in each assay.

Transiently transformed sunflower leaf discs (11 mm diameter) were obtained as described (Manavella and Chan, 2009). For each construct used, at least six discs cut from at least three different plants were analysed. To test the infiltration and transformation efficiencies, the expression of the simultaneously introduced kanamycin resistance gene was measured by real-time reverse transcription-PCR (RT-PCR) as described below.

Microarray set-up

Microarrays were based on the *Arabidopsis* Genome Oligo Set version 1.0 (Operon). This set consists of a total of 26 090 oligonucleotides that correspond to 22 361 annotated genes according to The *Arabidopsis* Information Resource (TAIR) genome annotation version 6. Microarrays were manufactured as previously described (Alves-Ferreira *et al.*, 2007).

Tissue collection and microarray experiments

Tissue collection for the different biologically independent sets of samples was done on different days but at the same time of day to minimize any diurnal effects on gene expression. Total RNA was isolated from all tissue samples using the Trizol reagent and the RNA was cleaned up with an RNeasy RNA isolation kit (Qiagen) according to the manufacturer's instructions. Dye-labelled antisense RNA was generated from these total RNA preparations and hybridized to microarrays using a MAUI hybridization system (BioMicro Systems) as previously described (Alves-Ferreira *et al.*, 2007). The dyes used for labelling RNA from the individual samples were switched in the replicate experiments to reduce dye-related artefacts.

Data analysis

Microarrays were scanned with an Axon GenePix 4200A scanner, using the Gene Pix 5.0 analysis software (Axon Instruments). Raw data were imported into the Resolver gene expression data analysis system (Rosetta Biosoftware) and processed as previously described (Alves-Ferreira *et al.*, 2007). The *P*-values calculated by this software were adjusted for each experiment using the Benjamini and Hochberg procedure as implemented in the Bioconductor multitest package (http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/multtest.html). Genes were considered as differentially expressed if they showed an absolute FC value of $\geqslant 2$ between the wild type and a mutant and had been assigned an adjusted *P*-value of 0.05. All analyses in Resolver were done at the so-called sequence level; that is, data from reporters (probes) representing the same gene were combined.

The percentage of promoters bearing the pseudopalindrome CAAT(C/G)ATTG was calculated by searching this sequence six times in 781 random chosen promoters in the TAIR9 database. Standard error was 0.1% taking these six samples.

Histochemical GUS staining

In situ assays of GUS activity were performed as described by Jefferson *et al.* (1987). Whole plants were immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-glucuronic acid solution in 100 mM sodium phosphate pH 7.0 and 0.1% Triton X-100, and, after applying a vacuum for 5 min, they were incubated at 37 °C overnight. Chlorophyll was cleared from the plant tissues by immersion in 70% ethanol.

RNA isolation and analysis by real-time RT-PCR measurements

Total RNA from *Arabidopsis* or sunflower plants (at the developmental stages indicated in the figure legends) for quantitative real-time PCR (qPCR) was prepared with Trizol reagent (Invitrogen, http://www.invitrogen.com/) following the manufacturer's instructions (Invitrogen). qPCRs were carried out using an MJ-Cromos 4 (Bio-Rad, Hercules, CA, USA) apparatus as previously described (Manavella *et al.*, 2006). The oligonucleotides used for these determinations are listed in Supplementary Table S2 available at *JXB* online.

Hormone treatments

Sunflower plants in the V2 and V4 developmental stage were sprayed with 100 μM SA, 200 μM JA, or left untreated (control). The hormone solutions were prepared in 0.2% Tween-20.

Pathogen infections

Pseudomonas syringae infections were carried out by spraying a suspension of virulent or avirulent strains (Pst DC3000 and Pst DC3000/avrRpt2, respectively) as described by Katagiri *et al.* (2002).

Wounding and insect bioassays

For the wounding treatments of sunflower leaves, one-half of the lamina was damaged by crushing with fine tweezers (~50% of the surface was damaged). For the wounding treatment of *Arabidopsis* plants, the leaf (one-third of the surface) was crushed with fine tweezers and the remaining two-thirds were used for analysis. Larval mass gain was determined by placing one *Spodoptera exigua* larva per *Arabidopsis* plant (transgenic or controls). The plants were replaced daily and larval mass was determined daily during a period of 5 d with a microbalance. These tests were repeated at least 10 times using three independent transgenic lines for each genotype. For each experiment, at least 30 larvae were used per genotype.

Phytohormone extraction and quantification

Phytohormone extraction and quantification was carried out as previously described (von Dahl *et al.*, 2007; Manavella *et al.*, 2008) on flowering plants.

Results

HAHB10 *transcripts accumulate in both vegetative and reproductive stages*

The sunflower *HAHB10* gene accelerates the transition from the vegetative to the reproductive stage, leading to a shortening of the plant's life cycle when ectopically expressed in *Arabidopsis* (Rueda *et al.*, 2005). In order to investigate the mechanisms underlying this process, the expression pattern of *HAHB10* was first analysed in different sunflower tissues and developmental stages. Figure 1A shows that this gene was almost constitutively expressed in leaves, stems, and shoot apexes during the vegetative stage in LD while an inductive condition (SD) strongly induced the expression in shoot apexes. *HAFT* (*HELIANTHUS ANNUS FLOWERING TIME*) and *HAAP1* (*HELIANTHUS ANNUUS APETALLA 1*) transcripts were identified by phylogenetic analysis (data not shown) and quantified as controls in the same samples (leaves, shoot apexes, and stems), showing a very similar pattern with an additional and expected induction of *HAFT* in leaves (Fig. 1B, C). *HAHB10* and *HAFT* also showed a similar pattern in floral organs, while *HAAP1*, as expected, did not show expression at all in these

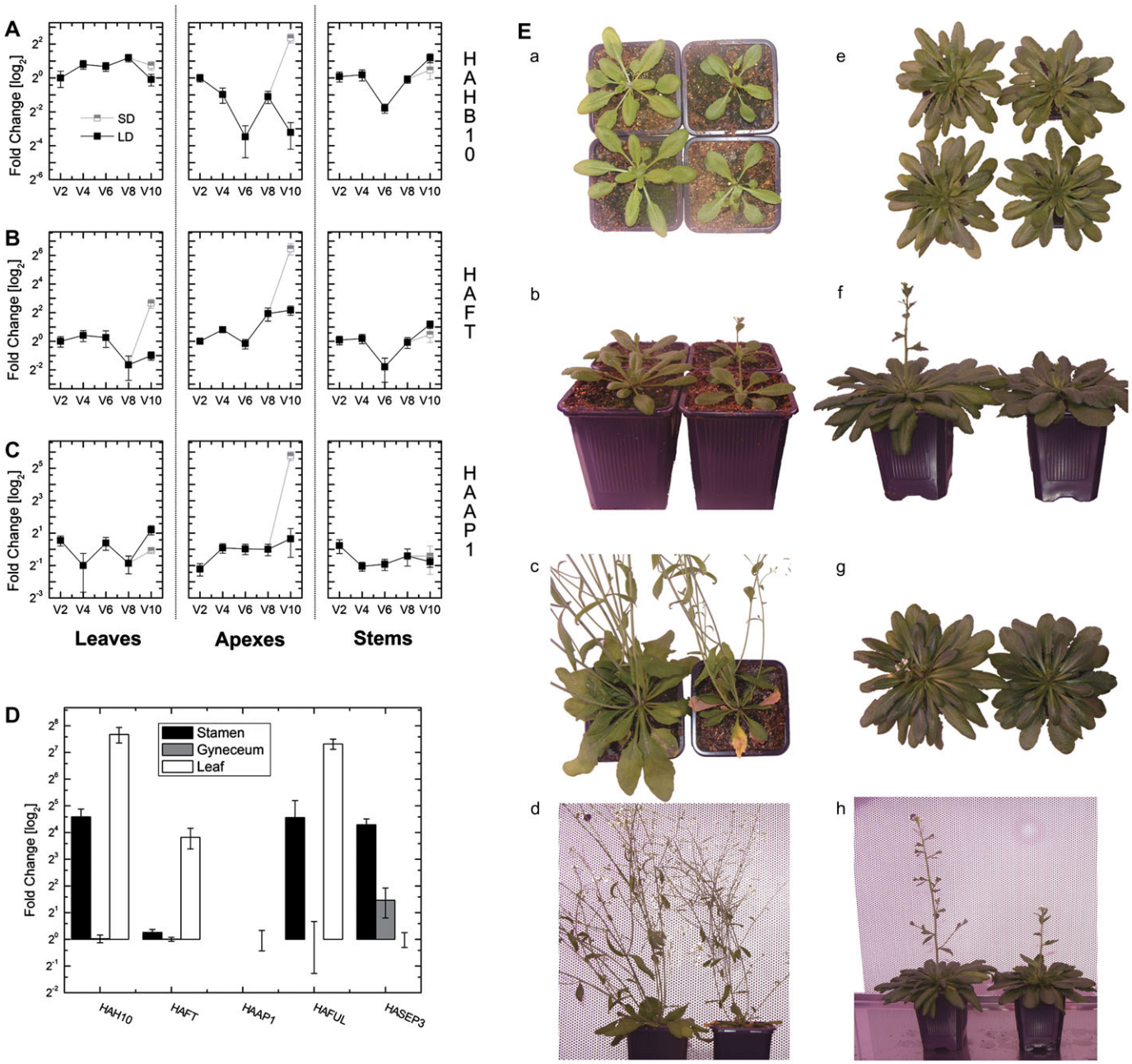

Fig. 1. Expression pattern of sunflower *HAHB10* during vegetative and reproductive stages. (A) *HAHB10* transcript levels in leaves (left panel), shoot apexes (central panel), and stems (right panel) at different developmental stages were measured by quantitative RT-PCR and related to the level quantified in young leaves (V2) arbitrarily taken as 1. Vx (vegetative stages from V2 to V10) in which x is a number representing the sunflower developmental stage according to Schneiter and Miller (1981). (B) Transcript levels of *HAFT1* quantified in the same samples. (C) Transcript levels of *HAAP1* quantified in the same samples. LD, plants grown in long-day conditions (16 h light/8 h darkness); SD, V10 plants grown in LD were placed under a 8 h illumination regime during 96 h prior to RNA isolation. (D) *HAHB10*, *HAFT*, *HAAP1*, *HAFUL*, and *HASEP3* transcript levels quantified in reproductive organs (stamens and gynoecium) at the R5 developmental stage. Quantitiative RT-PCR values are related to that obtained in the organ in which the expression was the lowest and this last value was arbitrarily taken as 1. Standard errors were calculated taking three independent biological samples in which actin transcripts (*ACTIN2* plus *ACTIN8*) were used as internal controls. Differences were considered significant when the *P*-values were <0.05 (Students *t*-test). (E) *35S:HAHB10* transgenic plants grown in LD (a, b, c, d) or SD (e, f, g, h) conditions compared with wild-type plants (*35S:GUS*) grown in the same conditions. In each panel, transgenic plants are on the right side while controls are on the left. a and b, 25-day-old plants; e and f, 50-day-old plants; c and d, 45-day-old plants; g and h, 60-day-old plants.

organs (Fig. 1D). In addition, *HASEP3* (*HELIANTHUS ANNUUS SEPALLATA 3*) and *HAFUL* (*HELIANTHUS ANNUUS FRUITFULL*) transcripts (identified by phylogenetic analysis) were quantified in these organs as controls, showing a similar pattern to their homologues from *Arabidopsis*.

In order to correlate the expression pattern of *HAHB10* to flowering, sunflower plants, responsive to SD, were placed in this condition when they reached the V10 stage. Notably, *HAHB10* expression was clearly induced together with *HAFT* and *HAAP1*, indicating that this gene may function in the transition from the vegetative to the reproductive stage.

The phenotype of *35S:HAHB10 Arabidopsis* transgenic plants in SD was analysed and, as is shown in Fig. 1E, a clear reversion of the flowering acceleration takes place in LD, supporting a specific role for HAHB10 in flowering induction depending on the photoperiod.

As a second approach to characterize the function of this gene, a 1399 bp fragment corresponding to the promoter region of *HAHB10* was isolated from a sunflower genomic library and inserted upstream of the *GUS* reporter gene to generate transgenic *Arabidopsis* plants. In seedlings, *GUS* expression was evident in cotyledons, mainly in the vasculature (Fig. 2A), while in the vegetative stage expression was detected in leaves (primarily in the secondary veins) and roots (Fig. 2A). *GUS* expression was not detected in the central vascular system but it was detected in primary buds and stems (Fig. 2A). During the reproductive stage, *GUS* expression was evident in stamens and in stigmatic papillae and style of carpels at late stages of flower development (Fig. 2A). Thus, a strong correlation was observed between expression of *HAHB10* in sunflower tissues and in *Arabidopsis* transgenic *promHAHB10:GUS* plants, suggesting the presence of conserved regulatory mechanisms for the expression of this gene in *Arabidopsis*.

Thirdly, to investigate whether the early flowering phenotype observed in *Arabidopsis* plants was the result of an indirect effect induced by the constitutive expression of *HAHB10* (*35S:HAHB10*), the *HAHB10* cDNA was cloned downstream of its native promoter and used to generate transgenic *Arabidopsis* plants (*promHAHB10:HAHB10*). There were no morphological differences between *promHAHB10:HAHB10* and control plants, whereas *35S-HAHB10* did show changes. When grown in LD, *prom-HAHB10:HAHB10* leaves were similar to wild-type leaves in shape, size, and number (Fig, 2C). Moreover, the darker green colour, observed in *35S:HAHB10 Arabidopsis* plants, was not evident in *promHAHB10:HAHB10* plants (Fig. 2C, lower panel). Both, *35S:HAHB10* and *promHAHB10:HAHB10* plants exhibited shorter life cycles compared with control plants (Fig. 2B and Table 1). In this regard, several

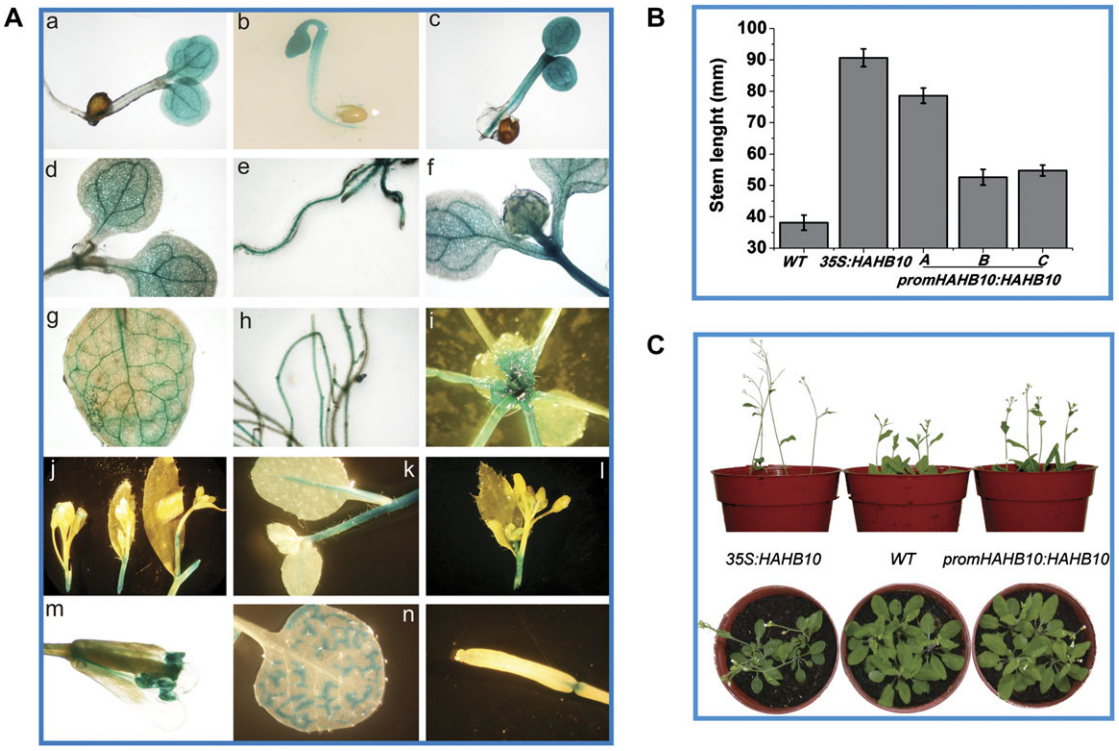

Fig. 2. *promHAHB10* directs the expression of *GUS* in different stages of development and promotes an early flowering in *Arabidopsis* plants transformed with *promHAHB10:HAHB10*. (A) Histochemistry of transgenic *Arabidopsis* plants expressing the *GUS* reporter gene under the control of the *HAHB10* promoter. (a, b, c) Three-day-old seedlings in control conditions, grown in darkness, and under far red illumination respectively. Ten-day-old aerial organs. (d, e, f) Seven-day-old plants; (g, h, i) 21-day old plants; (j) different stages of the inflorescences; (k) stems of a 35-day-old plant; (l, m) immature and mature inflorescences; (n) 35-day-old leaf; (o) 50-day-old plant silique. (B) Stem length (mm) measured with a ruler in 25-day-old plants from the three genotypes. (C) Front and upper view of three *Arabidopsis* transgenic genotypes. From left to right: *Arabidopsis* plants transformed with *35S:HAHB10*, control plants transformed with *35S:GUS*, and plants transformed with *promHAHB10:HAHB10*. This is a representative experiment performed with 32 individuals of each genotype.

Table 1. *PromHAHB10:HAHB10* plants have shorter life cycles compared with control plants

Phenotypic characteristics of transgenic plants expressing *HAHB10* under the control of its own promoter.

Genotype and line name	No. of plants per experiment	No. of rosette leaves	Bolting time (d)	Stem length of 25-day-old plants (mm)	No. of cauline leaves of 25-day-old plants	No. of silique leaves of 30-day-old plants	Plant age for harvest (d)
35S:GUS	32	10±1	21±1	38.12±2.4	2	2±1	65±2
35S: HAHB10-A	32	7±1	19±1	90.65±2.8	3	5±1	50±1
promHAHB10:HAHB10-A	32	9±1	20±1	78.59±2.4	3	4±2	57±2
promHAHB10:HAHB10-B	32	10±1	20±1	52.62±2.5	3	3±1	60±1
promHAHB10:HAHB10-C	32	10±1	20±1	54.75±1.7	3	3±1	59±2

Thirty-two individuals from each genotype as named in the first column were grown sharing the tray with an equal number of *35S:GUS* individuals, used as controls, under standard conditions as described in the Materials and methods. Phenotypic parameters were taken at the periods indicated in the respective columns. The number of rosette leaves was determined in the transition from vegetative to reproductive stage. The experiment was repeated at least three times with these lines and the data shown are the average of the replicate. Plants with a high level expression of *35S:HAHB10* were advanced in their development and attained their maximal heights earlier. These are representative examples of experiments using other transgenic lines for each construction showing similar results (not included). Standard errors are expressed as the ratio between standard deviations and the square of the number of independent measurements.

reproduction-associated developmental processes including bolting time, stem length, number of siliques, and silique maturation time were markedly different between the genotypes. The main difference was observed between control plants and *35S:HAHB10* plants, while *promHAHB10:HAHB10* plants showed intermediate values (Table 1). These results strongly suggested that the early flowering induced by the constitutive expression of *HAHB10* in *Arabidopsis* (Rueda *et al.*, 2005) was not an artefact of its ectopic expression.

The expression of HAHB10 *induces significant changes in the* Arabidopsis *transcriptome*

In order to investigate the mechanisms involved in the developmental phenotype conferred by *HAHB10*, a comparative transcriptome analysis of *Arabidopsis* transgenic (*35S:HAHB10*) and control plants was performed. From a total of 30 081 genes analysed, 781 showed altered expression levels after a Benjamin and Hochberg false discovery rate (BDH-FDR) correction and selection for *P*-values <0.05 (Supplementary Table S1 at *JXB* online). The differentially expressed transcripts belonged to several different metabolic and signalling pathways according to their gene ontogeny (GO) annotation (Al-Shahrour *et al.*, 2006). The signalling pathways exhibiting a significant overrepresentation were those of defence responses and flowering. A list of genes related to development and especially to flowering initiation, as well as those related to the photoperiod pathway, is presented in Table 2.

Validation of the microarray results for some of these genes was performed by qPCR on three biological replicates coming from independent transgenic lines. A good correlation in the changes of gene expression was found between the two methods (Tables 2, 3 and 4).

Like other members of the HD-Zip II subfamily, HAHB10 binds *in vitro* to the pseudopalindromic sequence CAAT(C/G)ATTG (Tron *et al.*, 2002). An investigation was therefore carries out to determine which of the differentially expressed genes contain this pseudopalindromic sequence in their promoter regions. For this analysis, a region of 1000 bp upstream of the transcription initiation site of the corresponding genes was extracted and evaluated [*Arabidopsis* promoters (TAIR9 genome release)]. Of the 781 differentially expressed genes, 2.8% contained the CAAT(C/G)ATTG element, indicating that this percentage of genes could be direct targets of HAHB10. This value was 1.1% higher than the value corresponding to the percentage of genes containing the promoter sequence [CAAT(C/G)ATTG] in the complete *Arabidopsis* genome (1.7%).

Early flowering is associated with up-regulation of a key set of genes in transgenic plants

Based on the early flowering phenotype conferred by *HAHB10* and the gene expression data, the analysis was focused on those genes involved in the transition from the reproductive to the flowering stage (which presented the largest changes in the microarray analysis) and their transcript levels were measured in 5-week-old leaves of *35S:HAHB10* transgenic plants grown in LD. *SEP3* transcript levels were induced ~25-fold in all the transgenic lines tested, whereas *FT* and *FUL* transcript levels were increased ~5-fold compared with control plants (Fig. 3). To understand better the numerical differences between the microarray and the qPCR results, it must be considered that these strong inductions were detected in leaves from mature plants (already in the reproductive–fructification stages) while the microarray analysis was performed with leaves from 21-day-old plants (entering the vegetative–reproductive stage).

To correlate the data obtained in *Arabidopsis* with the regulatory networks involving *HAHB10* in sunflower, the expression pattern of putative target genes, homologous to those identified as differentially expressed in *Arabidopsis*, was analyzed in transformed leaf discs. *HASEP3* and *HAFT* transcripts were evaluated in this tissue transformed with the construct *35S:HAHB10* or with the control construct *35S:GUS*. Consistent with the results obtained in *Arabidopsis*, overexpression of *HAHB10* induced *HASEP3* and *HAFT* genes ~15-fold.

Table 2. *Genes involved in flowering which change their transcript levels in transgenic plants expressing* HAHB10

ID	Description	Log_2 ratio	*P*-value
Data obtained in the microarray analysis			
At1g02230	*NAM* (no apical meristem)	−1.2	4.00E-02
At1g18810	Phytochrome kinase substrate 1	−0.99	1.00E-10
At4g32980	*ATH1* (HD-BELL homeobox protein)	−0.86	7.00E-04
At5g14920	Gibberellins-regulated protein 1 precursor	−0.61	6.00E-06
At2g43010	*PIF4* (phytochrome-interacting factor 4)	−0.52	3.00E-05
At5g62430	*CDF1* (cycling 2 factor 1)	−0.44	3.00E-02
At2g02950	*PKS1* (phytochrome kinase substrate 1)	−0.36	1.00E-02
AT1g14280	*PKS2* (phytochrome kinase substrate 1)	−0.35	6.00E-05
At1g75820	*CLV1* (CLAVATA 1 receptor kinase)	0.21	5.00E-02
At1g68050	*FKF1* (E3 ubiquitin ligase SCF complex F-box subunit)	0.24	2.00E-02
At1g14920	*GAI/RGA2* (GA insensitive-gibberellin response modulator)	0.3	2.00E-02
At1g22770	*GI* (gigantea protein)	0.31	1.00E-02
At1g56170	*HAP5* (CCAAT-box binding transcription factor Hap5 putative)	0.32	4.00E-02
At1g04400	*CRY2* (cryptochrome 2 apoprotein)	0.35	2.00E-02
At3g58070	*GIS* (GLABROUS INFLORESCENCE STEMS)	0.35	4.00E-02
At5g47640	*HAP3b* (CCAAT-box binding transcription factor Hap3b)	0.39	6.00E-05
At2g45660	*SOC1* (suppressor of overexpression of *CO* 1-*AGL20*)	0.44	9.00E-04
At5g15840	*CO* (zinc finger protein CONSTANS)	0.45	6.00E-02
At1g54830	*HAP5a* (CCAAT-box binding transcription factor Hap5a)	0.47	2.00E-03
At1g53160	*SPL4* (squamosa promoter-binding protein-like 4)	0.49	1.00E-02
At1g62360	*STM* (SHOOT MERISTEMLESS)	0.71	5.00E-07
At1g66350	*RGL1* (gibberellin regulatory protein)	0.79	7.00E-06
At3g54340	*AP3* (floral homeotic protein APETALA3)	0.91	2.00E-03
At5g03840	*TFL1* (terminal flower 1 protein)	1.05	6.00E-08
At4g08150	*KNAT1* (homeobox protein knotted-1 like 1)	1.14	6.00E-03
At1g65480	*FT* (flowering locus T protein)	1.25	1.00E-02
At5g24780	*VSP1* (vegetative storage protein 1)	1.28	2.00E-02
At1g74670	*GASA4* (gibberellins-regulated protein 4 precursor)	1.41	6.00E-09
At1g69600	*ATHB29* (ZF-HD homeobox family protein)	1.58	1.00E-02
At5g60910	*FUL* (MADS-box protein FRUITFULL)	1.83	—
At5g65080	*MAF5* (MADS AFFECTING FLOWERING 5)	1.85	1.00E-04
At3g02310	*AGL4* (floral homeotic protein)	2.2	—
At5g20240	*PI* (floral homeotic protein PISTILLATA)	2.46	8.00E-05
At5g15800	*AGL2* (floral homeotic protein)	2.61	—
At1g24260	*SEP3* (MADS-box protein)	3.37	—
Data obtained in quantitative RT-PCR			
At5g03840	*TFL1* (terminal flower 1 protein)	1.26	6.E-02
At1g65480	*FT* (flowering locus T protein)	1.68	1.E-02
At5g60910	*FUL* (MADS-box protein FRUITFULL)	1.64	1.E-02
At5g65080	*MAF5* (MADS AFFECTING FLOWERING 5)	1.41	5.E-02
At3g02310	*AGL4* (floral homeotic protein)	1.30	5.E-02
At5g20240	*PI* (floral homeotic protein PISTILLATA)	0.92	4.E-02
At1g24260	*SEP3* (MADS-box protein)	2.17	1.E-02
At5g15840	*CO* (zinc finger protein CONSTANS)	0.93	3.E-02

The first column shows gene identity; the second column shows the gene name and description; the third column shows the log2 of the ratio between transcript levels in transgenic (*35S:HAHB10*) plants related to those in control plants. The *P*-value was determined according to the Bonferroni test; – indicates that the *P*-value is $<E^{-10}$.

HAHB10 *down-regulates genes involved in the defence response*

A significant number of genes related to defence responses were identified as differentially expressed (most of them down-regulated) in transgenic plants constitutively expressing *HAHB10* (Table 3). Among them, there were some genes participating in the initial steps of the defence response such as *ICS2* (*ISOCHORISMATE SYNTHASE 2*) and *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY 1*), both required for SA synthesis/signalling in pathogen-challenged plants, and genes encoding pathogenesis-related proteins such as *PR2* and *PR5* (Table 3).

Some of these genes together with other defence marker genes were selected to analyse their expression by qPCR. *PR1* and *PDF1.2a* mRNA levels were repressed in *HAHB10*-expressing plants compared with control plants (Fig. 4A). The mRNA levels of *ICS1* (involved in SA

Table 3. *Genes involved in the defence response which change their expression level in plants transformed with* HAHB10

ID	Description	Log_2 ratio	*P*-value
Data obtained in the microarray analysis			
At1g02360	Chitinase putative	−2.33	—
At3g23220	*AP2/ATERF*	−1.83	3.E-02
At1g73330	*DR4* (protease inhibitor)	−1.69	—
At1g18870	*ICS2* (isochorismate synthase 2)	−1.66	2.E-03
At3g22231	*PCC1* (PATHOGEN AND CIRCADIAN CONTROLLED 1)	−1.52	5.E-02
At2g32680	*ATRLP23* (receptor like protein 23)	−1.51	6.E-09
At4g36470	Similar to SAM:JMT and to SAM:SAMT	−1.44	2.E-05
At3g05730	*DEFL* (defensin-like family protein)	−1.32	7.E-07
At1g72930	*TIR* (Toll-interleukin-resistance)	−1.31	1.E-03
At3g50470	*HR3* (hypersensitive response protein 3)	−1.25	2.E-02
At3g56400	*WRKY70* (WRKY family transcription factor 70)	−1.23	2.E-09
At1g02450	*NIMIN-1* (NPR1/NIM1-interacting protein 1)	−1.21	4.E-04
At3g25760	*AOC1* (allene oxide cyclase)	−1.09	2.E-02
At1g75040	*PR5* (pathogenesis-related protein 5)	−1.03	3.E-06
At3g48080	Disease resistance protein-related	−1.00	9.E-03
At3g25882	*NIMIN-2* (NPR1/NIM1-interacting protein 2)	−0.99	2.E-04
At1g33590	LRR protein-related similar to Hcr2-5D	−0.96	2.E-08
At3g23110	*AtRLP37* (disease resistance family protein similar to Cf-2.2)	−0.92	5.E-07
At1g17600	TIR-NBS-LRR class	−0.89	2.E-03
At5G48657	Defence protein-related	−0.88	4.E-02
At3g20600	*NDR1* (non-race-specific disease resistance protein)	−0.87	—
At1g72940	TIR-NBS class	−0.85	1.E-07
At2g40750	*WRKY54* (WRKY family transcription factor 54)	−0.85	6.E-07
At1g73325	Trypsin and protease inhibitor family protein	0.85	9.E-04
At3g13662	Disease resistance-responsive protein-related	0.88	3.E-02
At4g23600	*CORI3* (coronatine-responsive tyrosine aminotransferase)	0.93	7.E-04
At3g45140	*ATLOX2* (lipoxygenase 2)	1.02	1.E-03
At5g42500	Similar to disease resistance response protein 206-d	1.02	2.E-02
At2g42885	*DEFL* (encodes a defensin-like family protein)	1.23	2.E-03
At5g24780	*VSP1* (vegetative storage protein 1)	1.28	2.E-02
At1g19640	*JMT* (*S*-Ade-L-Met:JA carboxyl methyltransferase)	1.31	2.E-03
At4g10265	Similar to wound-induced protein of *L. esculentum*	1.41	1.E-02
Data obtained in quantitative RT-PCR			
At1g73330	*DR4* (protease inhibitor)	−1.41	5.E-02
At1g18870	*ICS2* (isochorismate synthase 2)	−0.93	1.E-02
At1g02450	*NIMIN-1* (NPR1/NIM1-interacting protein 1)	−1.63	5.E-02
At3g25760	*ERD12* (allene oxide cyclase)	−0.52	4.E-02
At3g25882	*NIMIN-2* (NPR1/NIM1-interacting protein 2)	−2.24	5.E-02

The first column shows gene identity; the second column shows the gene name and description; the third column shows the log2 of the ratio between transcript levels in transgenic (*35S:HAHB10*) plants related to those in control plants. The *P*-value was determined according to the Bonferroni test; – indicates that the *P*-value is $<E^{-10}$.

synthesis), *EDS5* (encoding a transporter in SA signalling), *AOC1* (involved in JA synthesis), and *PR2* (involved in the defence response) were also lower in these plants (Fig. 4). These results showed that the basal levels of expression of some genes involved in both SA and JA biosynthesis and some of their induced defence responses were affected in *35S:HAHB10 Arabidopsis* plants.

To assess whether the expression of these genes was also affected in sunflower leaves overexpressing *HAHB10*, their putative homologues in sunflower were identified by phylogenetic analysis (data not shown). The expression of *HAPR1*, *HAPR3*, and *HALOX2* transcripts was quantified in transiently transformed sunflower leaf discs. Consistent with the results obtained in *Arabidopsis*, overexpression of *HAHB10* repressed the levels of these mRNAs (Fig. 4B).

HAHB10 *is regulated by phytohormones and conditions related to biotic stresses*

In sunflower leaves, the expression of *HAHB10* and *HAFT* was induced after 12 h of SA treatment (Fig. 5A, B), whereas the expression of *HASEP3* did not change (Fig. 5C). *HAHB10* and *HAFT* presented similar kinetics of induction, with a peak at 12 h, slowly decreasing thereafter. The defence marker *HAPR1* presented different kinetics of induction, with a continuous increase up to 72 h (Fig. 5D). *HAHB10* was also induced after infection of sunflower leaves with

Table 4. *Arabidopsis* HD-Zip II members which change their transcript levels in transgenic plants expressing *HAHB10*

ID	Description	Log_2 ratio	*P*-value
Data obtained in the microarray analysis			
At4g17460	*HAT1* (homeobox-leucine zipper protein 1)	−2.35	—
At4g37790	*HAT22* (homeobox-leucine zipper protein 22)	−1.60	5.E-09
At4g16780	*HAT4* (homeobox-leucine zipper protein 4)	−1.58	3.E-06
At5g06710	*HAT14* (homeobox-leucine zipper protein 14)	−1.29	3.E-03
At5g47370	*HAT2* (homeobox-leucine zipper protein 2)	−1.18	—
At3g60390	*HAT3* (homeobox-leucine zipper protein)	−1.15	3.E-03
At2g22430	*ATHB-6* (homeobox-leucine zipper protein 6)	0.67	1.E-05
At2g46680	*ATHB-7* (homeobox-leucine zipper protein 7)	0.69	5.E-02
At4g40060	*ATHB-16* (homeobox-leucine zipper protein 16)	0.79	2.E-03
At5g65310	*ATHB-5* (homeobox-leucine zipper protein 5)	0.93	1.E-02
Data obtained in quantitative RT-PCR			
At4g17460	*HAT1* (homeobox-leucine zipper protein 1)	−1.31	1.E-02
At3g60390	*HAT3* (homeobox-leucine zipper protein 3)	−1.48	1.E-02
At4g16780	*HAT4* (homeobox-leucine zipper protein 4)	−2.20	1.E-02

The first column shows gene identity; the second column shows the gene name and description; the third column shows the log2 of the ratio between transcript levels in transgenic (*35S:HAHB10*) plants related to those in control plants. The *P*-value was determined according to the Bonferroni test; – indicates that the *P*-value is $<E^{-10}$.

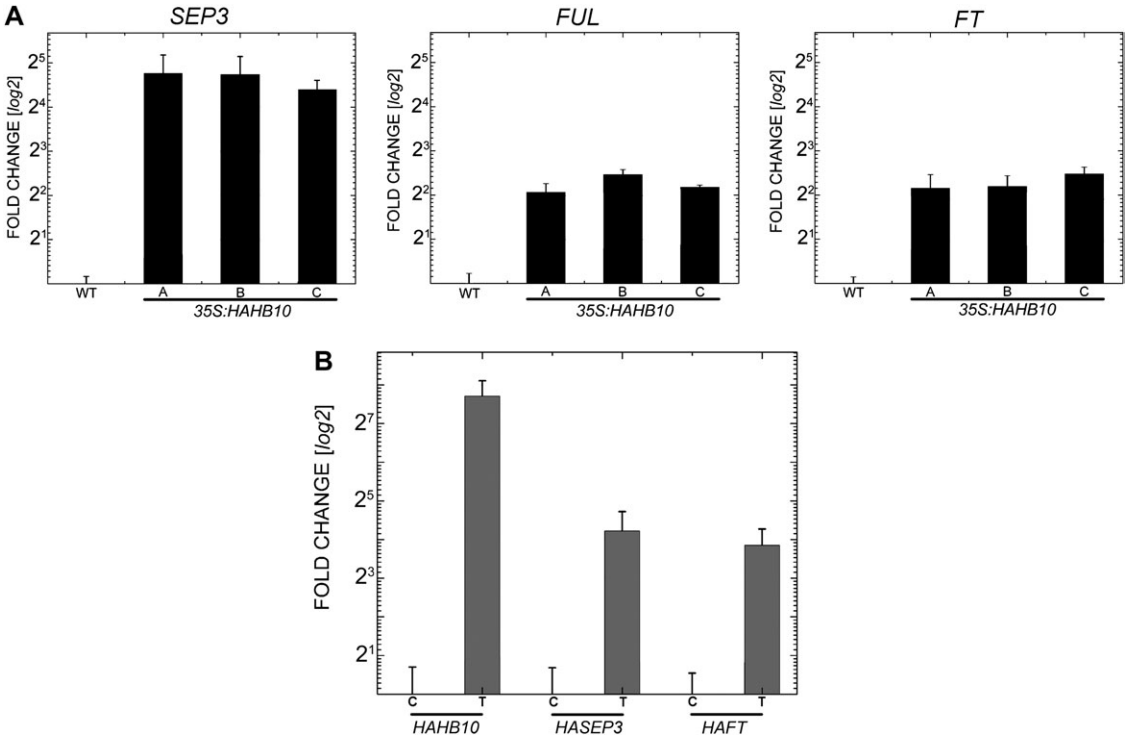

Fig. 3. Genes involved in flowering are induced in transgenic plants expressing *HAHB10*. (A) Transcript levels of *SEP3*, *FUL*, and *FT* genes, associated with flowering initiation, were analysed by qRT-PCR in three independent transgenic lines (A, B, and C) transformed with the construct *35S:HAHB10.* As controls, plants transformed with *35S:GUS* (named WT) were used. Transcript levels of each gene determined in three biological replicates were related to the level detected in WT leaves. *ACTIN* (*ACTIN2* plus *ACTIN8*) and *UBIQUITIN* (*UBI9*) were used as internal controls. Standard errors were calculated taking three independent experiments, and differences were considered significant when *P*-values were <0.05 (Students *t*-test). (B) Sunflower leaf discs were transformed with *35S:GUS* used as control (C) or with *35S:HAHB10* (T). Transcript levels of *HAHB10*, *HAFT* (DY917234.1), and *HASEP3* (EL489638.1) were measured by qRT-PCR 72 h after transformation. *ACTIN* (*ACTIN2* plus *ACTIN8*) and *UBIQUITIN* (*UBI9*) were used as internal controls. Standard errors were calculated from at least three independent experiments with biological sextuplicates, and differences were considered significant when the *P*-values were <0.05 (Students *t*-test).

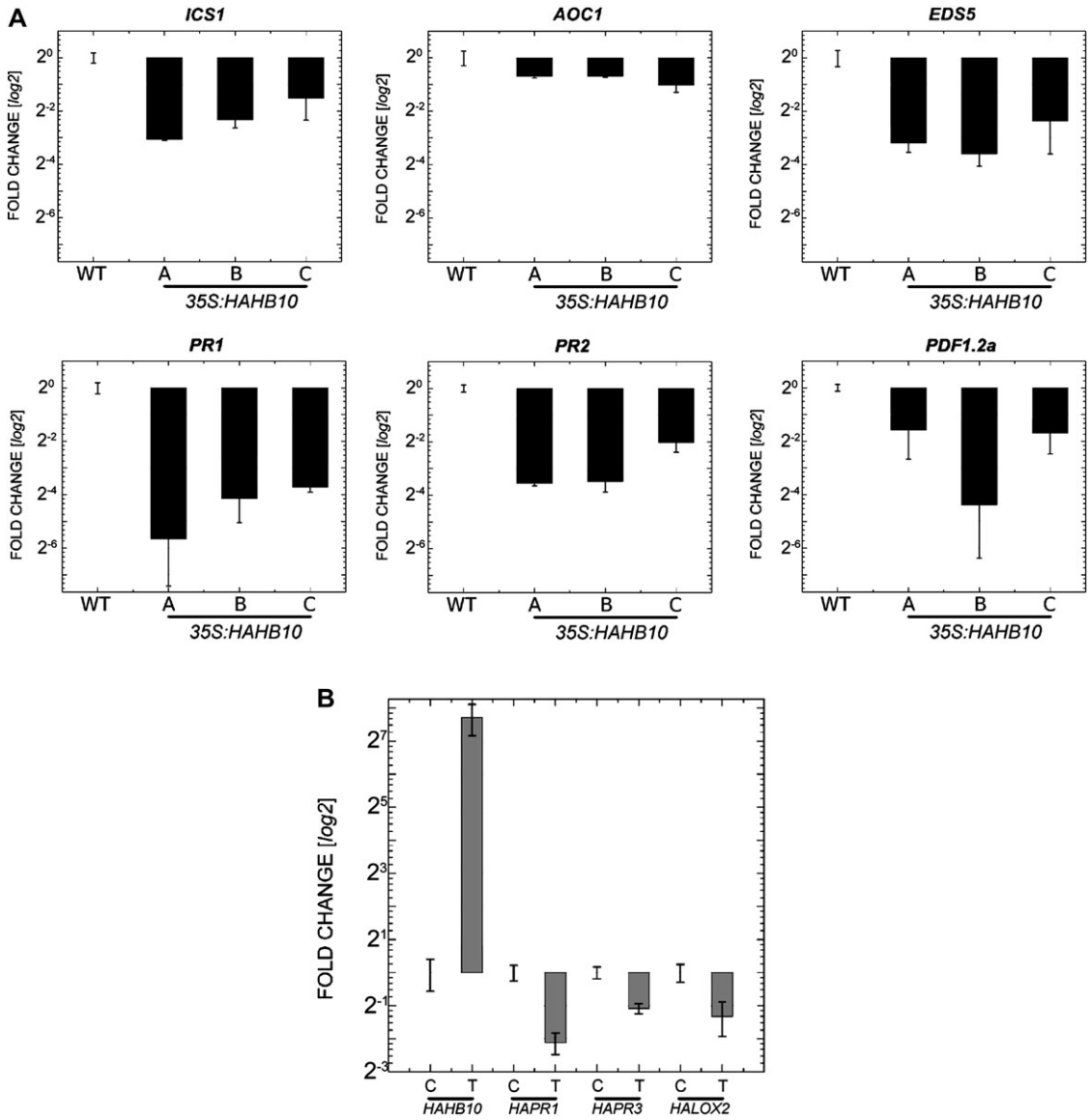

Fig. 4. Genes related to the biotic stress response are repressed in transgenic plants expressing *HAHB10*. (A) Transcript levels of *Arabidopsis ICS1*, *AOC1*, *EDS5*, *PR1*, *PR2*, and *PDF1.2* genes associated with the defence response were analysed by qRT-PCR in three independent transgenic lines (A, B, and C) transformed with the *35S:HAHB10* construct (*35S:HAHB10*). Control plants (WT) were transformed with pBI121. Quantifications were related to the level of each gene in WT leaves and repeated at least three times with biological triplicates. (B) Sunflower leaf discs were transformed with *35S:GUS* (C) or with *35S:HAHB10* (T). Transcript levels of *HAHB10*, *HAPR1*, *HAPR3*, and *HALOX2* were quantified by qRT-PCR 72 h after transformation. In both *Arabidopsis* and sunflower, A and B, *ACTIN* (*ACTIN2* plus *ACTIN8*) and *UBIQUITIN* (*UBI9*) were used as internal controls. Standard errors were calculated from at least three independent experiments with six biological replicates, and differences were considered significant when the *P*-values were <0.05 (Students *t*-test).

a virulent strain of *P. syringae*, showing a peak at 48 h (Fig. 5E). In contrast, when the sunflower leaves were wounded, a significant repression of *HAHB10* mRNA levels was observed (Fig. 5F).

Transgenic *Arabidopsis* plants (3 weeks old) expressing *HAHB10* under the control of its own promoter were treated with SA and the levels of *FT*, *PR1*, and *PDF1.2a* transcripts were quantified in leaves 6 h after the treatment. The results showed (Fig. 5G–I) that under control conditions the transcript levels of *FT* were higher in transgenic plants than in the wild type, both in the constitutive genotype and in *promHAHB10:HAHB10* plants, and did not change significantly after the treatment. Under control conditions, *PR1* transcript levels were repressed in both transgenic genotypes; however, they were induced to similar levels in control plants after SA treatment. In contrast, *PDF1.2a* transcript levels remained repressed in all genotypes.

Levels of SA and JA are affected in Arabidopsis HAHB10-*expressing plants after wounding and bacterial infection*

As mentioned above, the constitutive expression of *HAHB10* in *Arabidopsis* reduced the basal levels of expression of genes

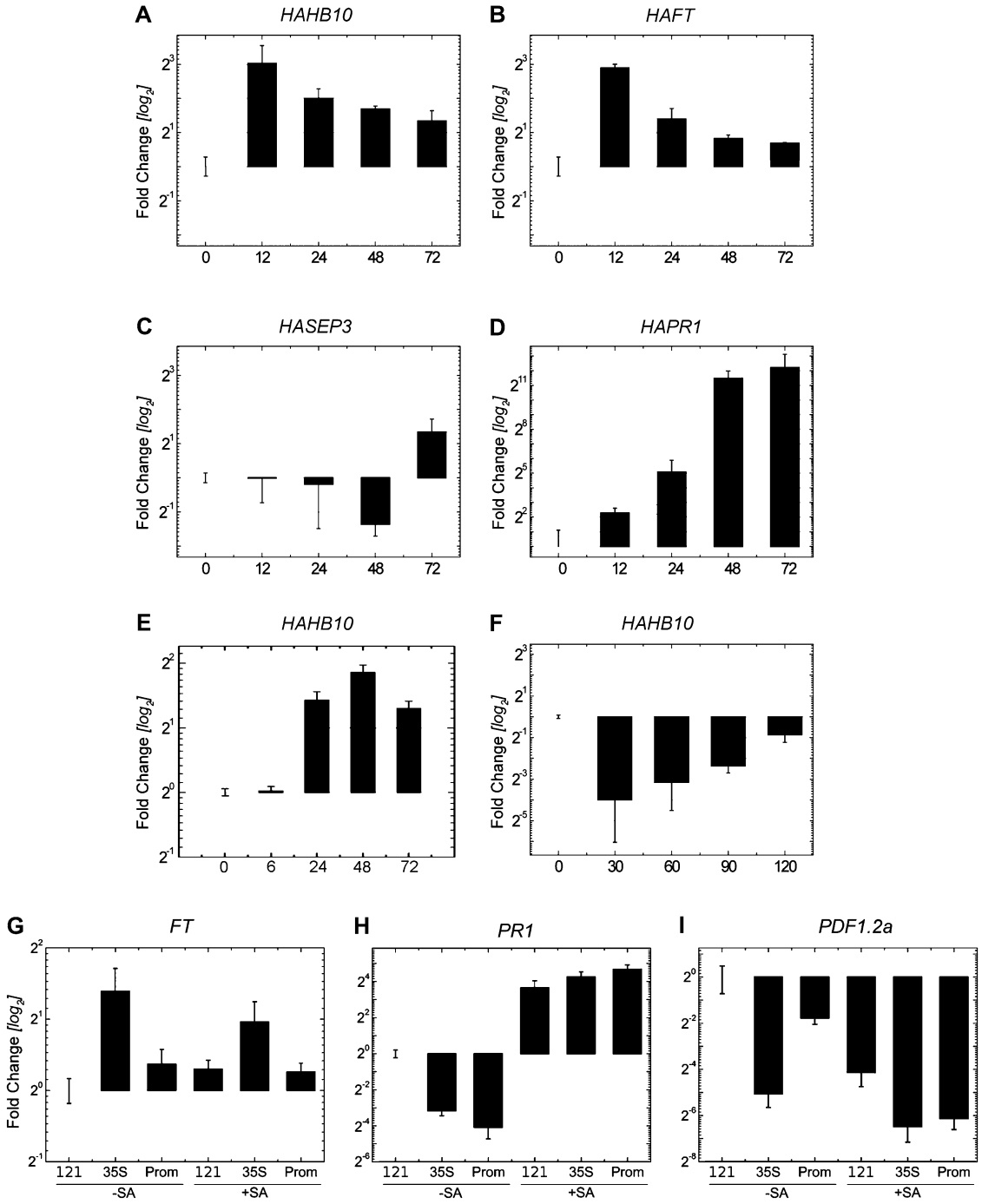

Fig. 5. *HAHB10* expression in sunflower is regulated by phytohormones and biotic stress. Kinetics of induction of *HAHB10* (A), *HAFT* (B), *HASEP3* (C), and *HAPR1* (D) with 100 μM SA treatment. Expression kinetics of *HAHB10* after infection with virulent (avir) and avirulent (vir) strains of *Pseudomonas syringae* DC3000. (F) Kinetics of *HAHB10* repression after wounding. The assays in A, B, C, and D were performed on V4 stage leaves. Time periods in which samples were collected are expressed in hours (A–E) or minutes (F). (G–I) Expression levels of *FT*, *PR1*, and *PDF1.2a* in 3-week-old transgenic (*35S:HAHB10* or *promHAHB10:HAHB10*) and WT (transformed with pBI 121) plants after a treatment with 1 mM SA during 6 h. *ACTIN* genes (*ACTIN2* plus *ACTIN8*) were used as internal controls. Standard deviations were calculated from at least three independent experiments with three biological replicates and differences were considered significant when the *P*-values were <0.05 (Students *t*-test).

regulated by SA and JA. Moreover, *HAHB10* mRNA levels were up-regulated by SA treatment but were, however, slightly repressed by wounding (Fig. 5). In order to elucidate some aspects of the complex relationship between *HAHB10* and SA- and JA-mediated responses, the amounts of these two phytohormones were quantified in *35S:HAHB10 Arabidopsis* plants and in control plants after mechanical damage and *P. syringae* infection.

The results indicated that the basal levels of JA and SA were similar between control and transgenic plants

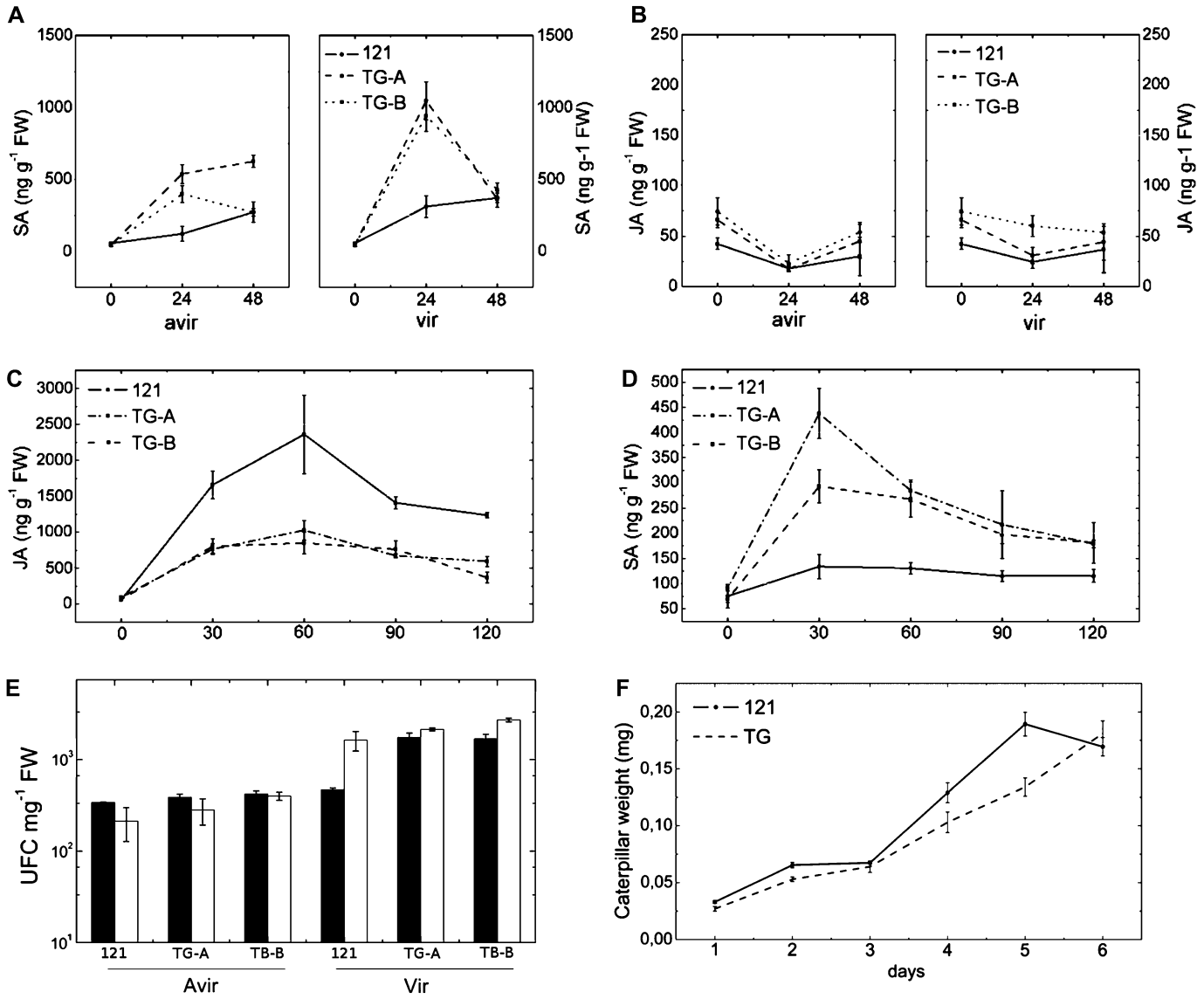

Fig. 6. The ectopic expression of *HAHB10* modulates the synthesis of SA and JA in *Arabidopsis* plants. (A and B) SA and JA quantification was performed in *Arabidopsis* plants transformed with *35S:HAHB10* (TG-A, TG-B) or with pBI121 (121, control). Levels of SA and JA were quantified after infection with an avirulent (Vir, left panel) or virulent (Avir, right panel) strain of *P. syringae*. Phytohormone levels were determined in leaves at 0, 24, and 48 h post-infection in four independent samples (n=4, bars: ±SD). (C and D) JA and SA quantification was performed in *Arabidopsis* plants transformed with *35S:HAHB10* (TG-A, TG-B) or with pBI121 (121, control) after 0, 30, 60, 90, and 120 min of mechanical wounding. Phytohormone levels were determined in leaves in four independent samples (n=4, bars: ±SD). (E) Bacterial colony-forming units (CFU) in *Arabidopsis* plants transformed with *35S:HAHB10* (TG-A, TG-B) or pBI121 (121, control). Bacterial density was quantified 24 h (white bars) and 48 h (black bars) post-infection with a virulent (Vir) and an avirulent (Avir) strain of *P. syringae*. Each determination was performed in triplicate. (F) Mass gain of *Spodoptera exigua* larvae on *HAHB10*-expressing *Arabidopsis* transgenic plants (TG) or plants transformed with pBI121 (121, control).

(Fig. 6A, 6B). However, 24 h after infection with a virulent strain of *P. syringae*, a significant increase in SA levels was observed in transgenic plants compared with control plants (Fig. 6A; all the differences were statistically significant; P <0.05, t-test). At 48 h, SA levels were reduced to control levels (Fig. 6A) while JA levels did not change significantly during infection (Fig. 6B). As expected, mechanical damage induced JA levels with a peak at 60 min in control plants; however, the levels were ~2.5-fold lower in transgenic plants than in controls (Fig. 6C; all the differences were statistically significant; P <0.05, t-test). In contrast, SA levels did not change significantly in wounded control plants whereas, similar to bacterial infection, they were elevated between 3- and 4.5-fold in wounded transgenic plants (Fig. 6D; all the differences were statistically significant; P <0.05, t-test).

Quantification of bacterial density [colony-forming units (CFU)] in leaves of plants infected with an avirulent and

a virulent strain of *P. syringae* showed that the avirulent strain grew at similar rates in control and transgenic plants whereas the virulent strain grew slightly faster (significant differences in CFU at 24 h); however, it reached similar CFU levels at 48 h of infection (Fig. 6E).

Because *Arabidopsis* plants ectopically expressing *HAHB10* also accumulated lower levels of JA after wounding compared with control plants, whether these plants were more susceptible to insect herbivores was also evaluated. First instar larvae of *S. exigua* were placed on transgenic and control plants and their gain in mass quantified every day for 6 d. The gain in mass of the larvae was similar between transgenic and control plants, indicating that the reduced levels of JA induced by the ectopic expression of *HAHB10* did not affect the susceptibility of the plants to *S. exigua* larvae (Fig. 6F).

Based on the effect of *HAHB4* on ethylene levels (Manavella *et al.*, 2008), the amount of ethylene released by *Arabidopsis HAHB10*-expressing plants and control plants was also quantified. The levels of this hormone were similar between genotypes (data not shown), indicating that, in contrast to *HAHB4*, ectopic expression of *HAHB10* in *Arabidopsis* does not affect ethylene levels.

Discussion

The expression pattern of HAHB10 *is consistent with the induction of early flowering*

HD-Zip transcription factors are usually expressed at very low levels; however, their expression increases as a result of specific external stimuli or internal signals (Ariel *et al.*, 2007). It has been shown that the expression of *HAHB10* is high in sunflower mature leaves, whereas it was almost undetectable in other tissues (Rueda *et al.*, 2005). A more extensive analysis performed in this study indicated that the expression of *HAHB10* in sunflower leaves was almost constitutive during the vegetative stage together with a lower expression of the newly identified *HAAP1* and *HAFT*. During the reproductive stage, *HAHB10* is expressed in stamen together with *HASEP3* and *HAFUL* but not in the gynoecium, while *HAAP1* transcripts disappear in both floral organs, as expected, and *HASEP3* is still present in the gynoecium.

Sunflower CF33 is an SD-responsive genotype while *Arabidopsis* Col 0 is LD responsive (de la Vega and Chapman, 2010). Interestingly, *HAHB10* as well as *HAFT* and *HAAP1* expression was strongly induced in apexes when sunflower plants were placed in an SD inductive condition while they remained almost constant in the non-inductive LD. In addition, when transgenic *35S:HAHB10* plants were grown in SD (non-inductive for *Arabidopsis*), *HAHB10* did not induce flowering. Moreover, the phenotype was reverted in this condition. These observations indicate that *HAHB10* action is photoperiod dependent, probably needing other photoperiod-dependent partners such as *CO* to exert its function. Similar observations were made for transcription factors belonging to the HAP family (Kumimoto *et al.*, 2008).

In general, the *GUS* expression pattern in leaves of *promHAHB10:GUS* plants was very similar to the reported expression pattern of *CO*, *FLC*, *FT*, and *FUL* in *Arabidopsis* (Mandel and Yanofski, 1995; Kardailsky *et al.*, 1999; Teper-Bamnolker and Samach, 2005; Kim *et al.*, 2008). Thus, the *GUS* expression pattern in *promHAHB10:GUS* plants was consistent with a potential function of *HAHB10* in regulating flowering by affecting the expression of key flowering genes (Fig. 2A).

Consistently, *Arabidopsis* transgenic plants transformed with a construct bearing this gene fused to its own promoter (*promHAHB10:HAHB10*), directing a tissue-specific expression, exhibited an early flowering and a shorter life cycle compared with control plants as was observed for constitutively *HAHB10*-expressing plants (*35S:HAHB10*) (Rueda *et al.*, 2005). This early flowering could be explained based on the high activity of the *HAHB10* promoter during the transition from the vegetative to the reproductive stage in the flower primordia, as it was observed in the *promHAHB10:GUS* transgenic plants. It is likely that the promoter responds to some environmental or internal signal and activates the transcription of *HAHB10*, thereby inducing the early flowering phenotype. Unfortunately, sunflower mutants (in this or other genes) are not available, precluding the analysis of the role of *HAHB10* in flowering regulation in this plant species. Accelerated flowering was also observed in transgenic plants ectopically overexpressing the *HAHB10* homologue, *ATHB2*/*HAT4* (Schena *et al.*, 1993), even though the phenotypic characteristics of *ATHB2*/*HAT4* ectopic overexpression and knock-out plants indicate that these two genes should not be considered as orthologues (Rueda *et al.*, 2005). However, another member of the *Arabidopsis* HD-Zip II family could be the actual orthologue of *HAHB10*. Based on the expression pattern, *HAT22* seems to be the best candidate (A. L. Arce *et al.*, unpublished results).

Analysis of gene expression supports a role for HAHB10 *in the induction of early flowering*

To understand the molecular mechanisms involved in the induction of flowering by *HAHB10*, the transcriptome of *35S:HAHB10* and control plants entering the flowering transition stage was compared. Several key genes involved in flowering initiation were found to be significantly up-regulated in *35S:HAHB10* plants, consistent with the early flowering phenotype induced by this transcription factor. For example, *SEP3* and *FUL* were described as key genes in floral organogenesis (Mandel and Yanofski, 1995; Pelaz *et al.*, 2001; Teper-Bamnolker and Samach, 2005). *FT* is considered the trigger of universal florigenic signals and regulates the flowering cycles in many plant species (Kardailsky *et al.*, 1999; Huang *et al.*, 2005; Lifschitz and Eshed, 2006; Lifschitz *et al.*, 2006). *SEP3* showed the highest induction in *HAHB10*-expressing plants, ~30-fold, and harboured in its promoter the pseudopalindrome

CAAT(A/T)ATTG, which binds HAHB10 *in vitro* (Tron *et al.*, 2002). Hence, *SEP3* may be a direct target of this HD-Zip. *FT* and *FUL* were also up-regulated in *35S:HAHB10* plants; however, these genes did not contain the CAAT(A/T)ATTG sequence in their promoter regions, suggesting that they are indirectly affected by HAHB10 expression. *CO* was slightly induced in the transgenic plants but to a lower level. In addition to flowering initiation genes, others, known to be involved in the photoperiod pathway, were also up-regulated. In agreement with the results obtained with *Arabidopsis*, putative sunflower homologues of *Arabidopsis FT* and *SEP3* (*HAFT* and *HASEP3*) were strongly induced when *HAHB10* was overexpressed in leaf discs. The high expression levels of *FT*, *SEP3*, and *FUL* were most probably sufficient to induce early flowering in *Arabidopsis* ectopically expressing *HAHB10*, since their overexpression generates a similar phenotype in this plant species (Kardailsky *et al.*, 1999; Pelaz *et al.*, 2001; Jaeger and Wigge, 2007).

The microarray analysis also revealed that transcript levels of *Arabidopsis* HD-Zip II members homologous to *HAHB10* were reduced in the *HAHB10* transgenic plants (Table 4). These results were in accordance with the already described negative autoregulation of HD-Zip II members (Ohgishi *et al.*, 2001; Ciarbelli *et al.*, 2008) and further suggested that HAHB10 was recognized in *Arabidopsis* as a functional HD-Zip II member.

Regarding these results indicating that *HAHB10* is involved both in flowering and in the defence response, *HAHB10* expression was analysed when sunflower plants were treated with exogenous SA, infected with *P. syringae*, or subjected to wounding. The expression of this gene was induced by SA, presenting similar kinetics to those of SA induction of *HAFT*, while *HASEP3* transcripts remained almost constant 72 h after the treatment. This last result is not in accordance with the up-regulation of *HASEP3* observed in transiently *HAHB10*-transformed leaf discs, suggesting that the concentration of *HAHB10* reached 72 h after SA treatment was not enough to induce *HASEP3* expression or, alternatively, that *HASEP3* is induced by *HAHB10* via an SA-independent pathway.

The accumulation of SA and JA is affected in Arabidopsis *plants constitutively expressing* HAHB10 *after infection with* P. syringae *and wounding*

Several defence-related genes were down-regulated in *35S:HAHB10* plants (Table 3). Among these genes were *ICS1* and *EDS5*, involved in SA synthesis and signalling, respectively, and *PR1* and *PDF1.2a* (Fig. 4). Moreover, the sunflower homologues of *PR1*, *PR3*, and *LOX2* (*HAPR1*, *HAPR3*, and *HALOX2*, respectively) were repressed at the mRNA level when *HAHB10* was transiently over-expressed in sunflower leaf discs. This general down-regulation of SA- and JA-dependent defence-related genes could be associated with a negative role for *HAHB10* in the regulation of SA and JA biosynthesis or their induced defence responses. Plants ectopically expressing *HAHB10* exhibited the same basal SA levels as their controls but accumulated more SA after a compatible interaction with *P. syringae* or wounding. Virulent bacteria showed an initial accelerated growth on *HAHB10*-expressing plants, in agreement with the basal reduced expression of some SA-responsive genes in these plants. However, after 48 h the bacterial density was similar to that of control plants, suggesting that the activation of SA-mediated responses was not impaired in *HAHB10*-expressing plants (consistent with the wild-type levels of activation of *PR1* gene expression after application of exogenous SA in this genotype). The increased accumulation of SA after infection could be the result of a compensatory effect for the reduced basal defence gene expression or a more direct de-regulation of SA biosynthesis or metabolism in *HAHB10*-expressing plants. The actual mechanism remains at present unknown. Prithiviraj *et al.* (2005) showed that SA is able to attenuate *P. aeuroginosa* virulence via the transcriptional repression of exoproteins and other virulence factors; however, this hormone did not inhibit bacterial growth.

The lower than control levels of JA (and JA–Ile) quantified in wounded and infected leaves of *35S:HAHB10* plants could be the result of a negative effect of the increased SA levels (Fig. 6). These reduced JA levels did not, however, affect the growth of larvae of the folivorous insect *S. exigua*. A related, however opposite, mechanism was described previously for the sunflower *HAHB4* (Manavella *et al.*, 2008); *Arabidopsis* plants ectopically expressing this transcription factor accumulated higher levels of JA and ethylene after wounding but reduced levels of SA after bacterial infection. Plants expressing *HAHB4* were more and less resistant to bacterial pathogens and insect herbivores, respectively (Manavella *et al.*, 2008). These results indicated that both *HAHB10* and *HAHB4* participate in the control of phytohormone synthesis and in signalling pathways affecting biotic stress responses (this study and Manavella *et al.*, 2006, 2008).

Conclusion

The results presented in this study indicated that *HAHB10* plays two different roles in plants. It induces the transition from vegetative to flowering stages via the activation of specific flowering transition genes in a photoperiod-dependent way and it affects the accumulation of SA and JA during a compatible interaction with *P. syringae* and wounding. A proposed model schematizing the participation of this transcription factor in these pathways is represented in Fig. 7. Whether the role of *HAHB10* in early flowering is associated with the regulation of SA biosynthesis/signalling remains a possible scenario and it is the topic of future work. Moreover, future experiments will also investigate the mechanisms induced by *HAHB10* that affect phytohormone accumulation during the wound response and defence against virulent *P. syringae*.

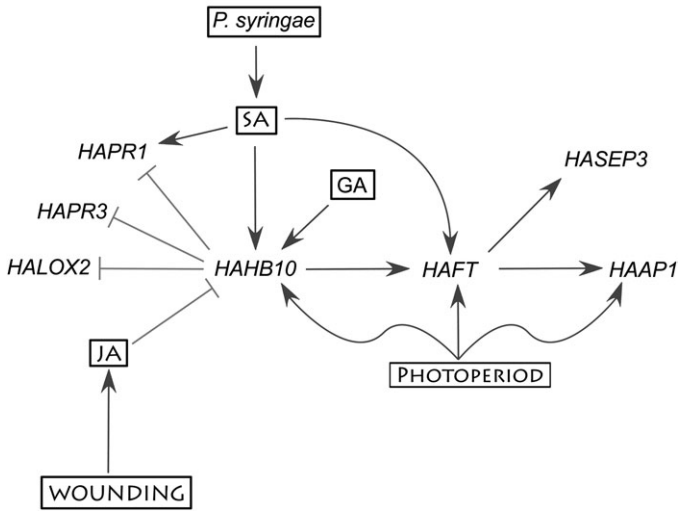

Fig. 7. Schematic model illustrating the role of *HAHB10* in flowering and biotic stress response. (→) indicates induction while (—|) indicates repression.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Microarray analysis of transgenic plants (21 days old) expressing *HAHB10*.

Table S2. List of oligonucleotides and gene IDs used for qRT-PCR determinations,

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