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Hahb-10, a Sunflower Homeobox-Leucine Zipper Gene, is Regulated by Light Quality and Quantity, and Promotes Early Flowering when Expressed in Arabidopsis

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Homeodomain-leucine zipper proteins constitute a family of transcription factors found only in plants. Expression patterns of the sunflower homeobox-leucine zipper gene *Hahb-10* (*Helianthus annuus* homeobox-10), that belongs to the HD-Zip II subfamily, were analysed. Northern blots showed that *Hahb-10* is expressed primarily in mature leaves, although expression is clearly detectable in younger leaves and also in stems. Considerably higher expression levels were detected in etiolated seedlings compared with light-grown seedlings. Induction of *Hahb-10* expression was observed when seedlings were subjected to treatment with gibberellins. Transgenic *Arabidopsis thaliana* plants that express *Hahb-10* under the 35S cauliflower mosaic virus promoter show special phenotypic characteristics such as darker cotyledons and planar leaves. A reduction in the life cycle of about 25% allowing earlier seed collection was also observed, and this phenomenon is clearly related to a shortened flowering time. When the number of plants per pot increased, the difference in developmental rate between transgenic and non-transformed individuals became larger. After gibberellin treatment, the relative difference in life cycle duration was considerably reduced. Several light-regulated genes have been tested as possible target genes of *Hahb-10*. One of them, *PsbS*, shows a different response to illumination conditions in transgenic plants compared with the response in wild-type plants while the other genes behave similarly in both genotypes. We propose that *Hahb-10* functions in a signalling cascade(s) that control(s) plant responses to light quality and quantity, and may also be involved in gibberellin transduction pathways.

Keywords: Gibberellin — *Hahb-10* — HD-Zip — Light-regulated expression — *PsbS* — Sunflower homeodomain-leucine zipper.

Abbreviations: HD, homeodomain; Zip, leucine zipper; GA_3, gibberellic acid.

Introduction

Several families of plant proteins contain a conserved DNA-binding motif known as the homeodomain, a 60 amino acid sequence present in a number of eukaryotic transcription factors involved in developmental processes. In one of these plant families, named HD-Zip, the homeodomain is associated with a leucine zipper dimerization motif. This family can be divided into several subfamilies according to sequence homology in and outside of the homeodomain. HD-Zip proteins, unique to plants, are proposed as good candidates to trigger developmental responses to changes in environmental conditions, a characteristic feature of plants. Several authors have reported that expression of members of the HD-Zip family of transcription factors is regulated by different external factors such as illumination or water stress (Schena and Davis 1992, Carabelli et al. 1993, Schena et al. 1993, Söderman et al. 1994, Carabelli et al. 1996, Söderman et al. 1996, Chan et al. 1998, Lee and Chun 1998, Söderman et al. 1999, Gago et al. 2002).

The HD-Zip II subfamily comprises nine members in *Arabidopsis thaliana* (Morelli and Ruberti 2002). The expression of a member of *Arabidopsis* subfamily II, *Athb-2/HAT4*, is regulated by far-red light and its function is related to shade avoidance responses (Carabelli et al. 1993, Steindler et al. 1999, Morelli and Ruberti 2000, Morelli and Ruberti 2002). Transgenic plants bearing constructs that alter the expression of *Athb-2/HAT4* exhibit changes in morphology and developmental rate (Carabelli et al. 1993, Schena et al. 1993). Plants expressing a *HAT4/Athb-2* antisense construct are shorter and develop more slowly than normal, whereas those expressing a *HAT4/Athb-2* sense construct show a shade avoidance phenotype characterized by elongated hypocotyls and petioles, as well as earlier flowering. Schena et al. (1993) concluded that *HAT4/Athb-2* functions as a master regulator of the developmental rate. These observations are reminiscent of those that accompany environmental stimuli such as signalling by light. They can be considered as adaptive responses also observed in other non-motile organisms (Gimeno et al. 1992).

HAT2, another member of *Arabidopsis* subfamily II, has been characterized as an auxin-inducible gene by DNA microarray screening (Sawa et al. 2002). *HAT2*-overexpressing plants that produce long hypocotyls, epinastic cotyledons, long

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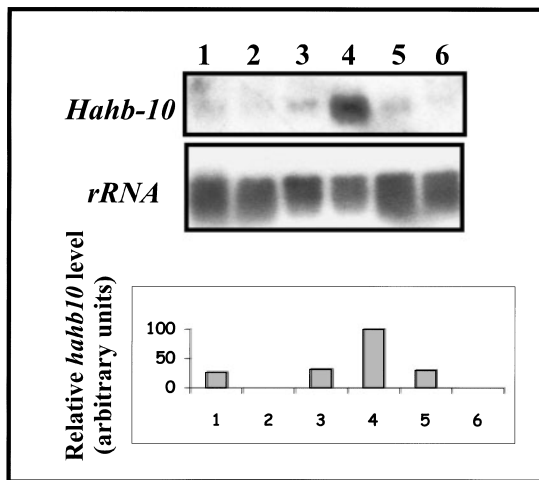

Fig. 1 *Hahb-10* is mainly expressed in mature leaves. Total RNA samples (10 μg each) extracted from 4-day-old seedlings (1), 14-day-old roots (2), 14-day-old stems (3), 30-day-old leaves (4), carpels (5) or fertile flowers (6) were hybridized with a ^{32}P-labelled *Hahb-10* cDNA-specific probe (upper panel) or with an rRNA probe used as control. The graphic in the lower panel shows *Hahb-10* transcript levels relative to the level in leaves, taken as 100%.

petioles and small leaves (typical characteristics of some auxin-overproducing mutants, Delarue et al. 1998) were obtained by these authors. These plants show reduced auxin sensitivity compared with wild-type plants. On the other hand, these transgenic plants showed reduced lateral root elongation. These observations led Sawa et al. (2002) to suggest that *HAT2* plays opposite roles in the shoot and root tissues in regulating auxin-mediated morphogenesis.

We have previously reported the isolation and characterization of several homeobox genes from sunflower (Gonzalez and Chan 1993, Chan and Gonzalez 1994, Gonzalez et al. 1997, Valle et al. 1997). *Hahb-4* is a member of subfamily I and its function is related to the response to water stress (Gago et al. 2002, Dezar et al. 2005).

Here we report expression and functional studies on a member of the sunflower Hd-Zip II subfamily, *Hahb-10*. Previously described experimental results suggest that redox conditions may operate to regulate the activity of this and other members of this subfamily (Tron et al. 2002). The studies performed here indicate that *Hahb-10* is expressed primarily in mature photosynthetic tissues, and is up-regulated by etiolation and gibberellins in seedlings. We have observed that *Arabidopsis* plants that overexpress *Hahb-10* have a characteristic phenotype that affects leaf shape, form and colour, growth

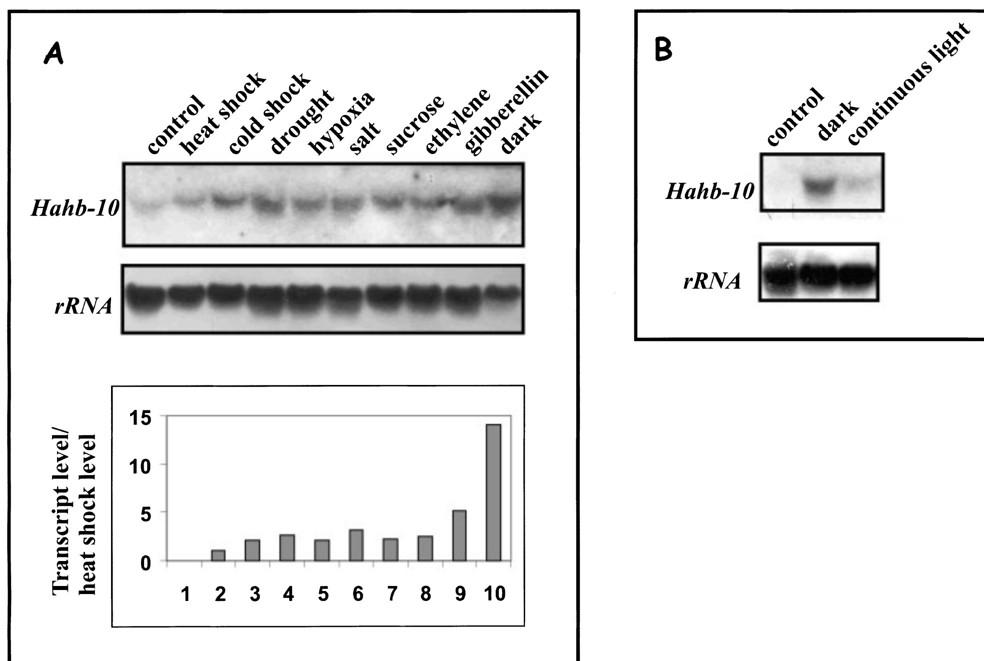

Fig. 2 *Hahb-10* expression is strongly induced in etiolated seedlings. (A) Total RNA samples (20 μg each) extracted from 4-day-old seedlings subjected to different treatments as described in Materials and Methods were hybridized with a ^{32}P-labelled *Hahb-10* cDNA-specific probe (A, upper panel) or with an rRNA probe used as control. The graphic shown in the lower panel shows *Hahb-10* transcript levels relative to the level measured in heat shock-treated seedlings. Quantitation of the signals has been done taking as standard value the signal obtained in these seedlings because the signal obtained in control seedlings was almost undetectable by the informatic program used for this purpose. (B) Total RNA samples (20 μg each) extracted from 7-day-old seedlings grown under normal illumination conditions (lane 1), in the dark (lane 2) or under continuous illumination applied to 4-day-old seedlings germinated in the dark during an additional 3 d (lane 3) were hybridized with a ^{32}P-labelled *Hahb-10* cDNA-specific probe (upper panel) or with an rRNA probe used as control (lower panel).

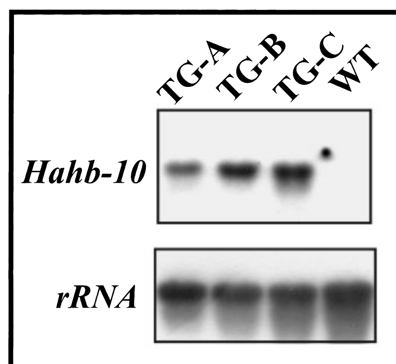

Fig. 3 Expression of *Hahb-10* in *Arabidopsis* transgenic plants. Northern blot analysis of transgenic Arabidopsis plants. Total RNA (10 μg) was extracted from wild-type (WT) and three independent transgenic plants (TG-A, -B and -C) overexpressing *Hahb-10*. Probes specific for *Hahb-10* or rRNA were used.

rate, flowering time and life cycle under standard growth conditions. Transgenic plants also show altered responses to changes in illumination quality and intensity. We propose that the product of this gene is involved in light-dependent responses related to plant development.

Results

Hahb-10 is primarily expressed in mature leaves

To perform expression analysis, we have used a specific probe that contains the 5′ portion of *Hahb-10*. Northern blot analysis using this probe with total RNA extracted from different sunflower organs showed a high expression level in 30-day-old leaves and lower levels in seedlings, stems and cotyledons (Fig. 1). Lower but detectable levels of the transcript were observed in roots, carpels and fertile flowers. Quantitation of the signals indicated that expression in mature leaves is 4- to 5-fold the level of that found in seedlings. The results indicate that this transcription factor may have a function during vegetative/reproductive developmental states in photosynthetic tissues.

Hahb-10 expression is regulated by light

Since *Hahb-10* belongs to the HD-Zip family, and members of this family are supposed to be involved in developmental processes regulated by external factors and/or hormones, we have investigated this possibility subjecting sunflower plants to different treatments. Fig. 2A shows the effect of such treatments on *Hahb-10* transcript levels. Heat or cold shock, water stress and hypoxia slightly enhanced *Hahb-10* mRNA levels. Addition of sucrose (10% w/v) or 30 mM Ethrel produced a similar effect, while 100 μM gibberellic acid (GA_3) raised *Hahb-10* transcript levels 5-fold with respect to the heat shock treatment. The strongest induction (14-fold) was observed when seedlings were grown in the dark (etiolated) during 7 d, indicating that the expression of this gene may be regulated by illumination conditions.

In order to investigate this point, plants were subjected to different illumination conditions: continuous light or darkness. Fig. 2B shows that *Hahb-10* expression is high in the dark, and extremely low under continuous illumination.

Table 1 Roots are shorter in transgenic than in non-transformed plants

Root length	WT plants	TG line A	TG line B	TG line C
Average ± SD	17.7 ± 2.9	14.8 ± 1.8	13.7 ± 2.2	12.7 ± 2.2

Root length in 8-day-old *Hahb-10*-overexpressing (TG line A, B, C) or non-transformed plants (WT) grown on Petri dishes under normal conditions (described in Materials and Methods) was measured with a ruler. This is a representative experiment done with 20 plants of each genotype. Root length is expressed in mm.

Table 2 *Hahb-10* transgenic plants have a higher concentration of Chl and anthocyanin than non-transformed plants

Quantitated pigment	WT	TG-A	TG-B	TG-C
Chl a	71.6 ± 1.1	79.8 ± 1.1	84.9 ± 1.5	88.0 ± 1.5
Chl b	27.6 ± 1.3	38.0 ± 1.4	40.2 ± 1.4	42.1 ± 1.4
Anthocyanin	0.06 ± 0.01	0.14 ± 0.02	0.15 ± 0.02	0.22 ± 0.02

Pigments were quantitated in *Hahb-10*-expressing transgenic plants (TG-A, TG-B and TG-C) and non-transformed Arabidopsis (WT) as described in Materials and Methods. Values are expressed as μg of pigment 100 mg of processed tissue. This is a representative experiment done with 20 plants of each genotype.

Obtaining Hahb-10 overexpressing Arabidopsis lines

To investigate the in vivo function of *Hahb-10*, we have used an overexpression approach. The coding region of *Hahb-10* was fused to the 35S promoter of cauliflower mosaic virus, and the construct was used to transform *Arabidopsis* plants. Several homozygous lines were recovered and, after preliminary analysis, three transgenic independent lines, named *35S: Hahb-10*-A, -B and -C (named TG-A, TG-B and TG-C, respectively, in figures and tables), were selected for more detailed analysis. Fig. 3 shows a Northern blot hybridized with the 5′ region of *Hahb-10*, that does not include the HD-Zip domain-coding region, where total RNA from non-transformed or transgenic plants was analysed. The probe did not hybridize with RNA extracted from wild-type plants, indicating the absence of cross-reactions with members of the *Arabidopsis* HD-Zip family or with other genes. Signals of variable strength were observed with RNA from the different transgenic lines, suggesting that they express *Hahb-10* mRNA at different levels. We have also tested if *Arabidopsis* homologues of this gene change their expression level in transgenic plants. Neither *HAT22* nor *Athb-2/HAT4* transcript levels were affected by the presence of the transgene (data not shown).

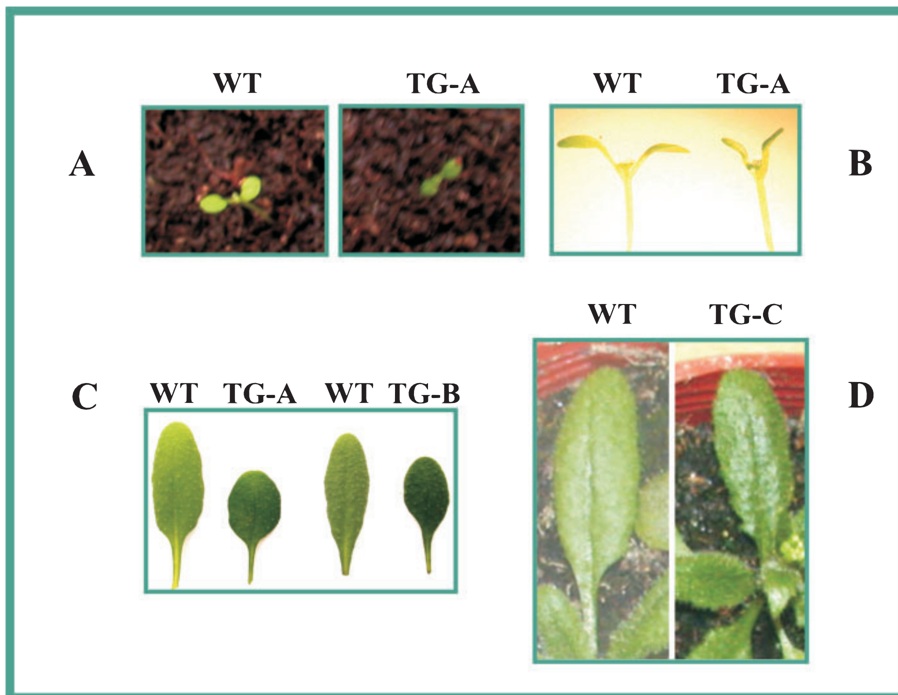

Fig. 4 Phenotype of *35S:Hahb-10* transgenic plants. Comparison between transformed and control plants. (A) Top view of cotyledons; (B) side view of cotyledons, (C) and (D) 21-day-old-leaves of the four genotypes (WT and TG-A, -B and -C).

Effect of illumination conditions on the phenotype of Hahb-10-overexpressing lines

Compared with wild-type plants, *35S-Hahb-10* transgenic plants exhibited a characteristic phenotype when cultured under standard conditions. When grown on soil, root length was shorter than in their wild-type counterparts (Table 1). Cotyledons were less expanded and remained at a smaller angle with respect to the hypocotyl axis, while in non-transformed plants, as expected, cotyledons were positioned perpendicular to the hypocotyl (Fig. 4A, B). In addition, all photosynthetic tissues presented a darker green colour. This darker colour is due to a higher concentration of total Chl and anthocyanin (Table 2). Leaves were smaller and planar in the transgenic lines compared with those of the non-transformed plants during vegetative and reproductive stages (Fig. 4C, D).

The observations made on the phenotype of *Hahb-10*-overexpressing lines suggest that they may have altered developmental responses to light. In order to investigate this, we have grown transgenic plants and non-transformed plants at different light intensities. As it can be observed, at low light intensities (upper panel of Fig. 5), transgenic plants show a defect in hypocotyl elongation. Hypocotyl elongation is a typical response observable in etiolated wild-type plants. In a similar way, a slight decrease in light intensity did not affect transgenic plant development (Fig. 5). While 15-day-old non-transformed plants have an average of 11.0 ± 0.6 mm in hypocotyl length when subjected to a light intensity of 35 μE m^{-2} s^{-1}, transformed plants show a hypocotyl length of 7.0 ± 0.5 mm on average. A higher but still low light intensity of 56 μE m^{-2} s^{-1} produced an attenuated effect: a hypocotyl length average of 4.2 ± 0.3 mm for 10-day-old wild-type plants and a value of 3.1 ± 0.3 mm for transgenic plants. Under far-red-enriched light, transgenic plants show reduced hypocotyl elongation (about 50%) compared with wild-type plants, while under red-enriched light transgenic hypocotyls are still shorter than wild-type hypocotyls (about 80%) but the difference between them is less pronounced (Fig. 5B).

Otherwise, transgenic plants are more tolerant to prolonged incubation in the dark. We have observed that the percentage of plants surviving after 5 d of etiolation and then subject to a normal illumination regime for an additional 4 d was only 18% in the non-transformed group compared with 75% survivors in the transgenic group. The number of survivors in the wild-type group increased when the dark treatment was applied during 3 d instead of 5 d to 37%, while a similar percentage of survivors with respect to a control group subjected to a normal illumination regime was observed in the transgenic group.

These results indicate that *Hahb-10*-overexpressing lines are less sensitive to changes in light conditions.

Transgenic Arabidopsis plants flower earlier and show a shorter life cycle compared with wild-type plants

Flowering occurs earlier in transgenic plants, when about seven rosette leaves are visible, while about 10 rosette leaves are present in wild-type plants when the inflorescence primordium appears. Transgenic plants showed a faster stem elongation rate than wild-type plants (Fig. 6). The differences in the stem height between wild-type and transgenic plants became less evident upon progression of the reproductive stage of

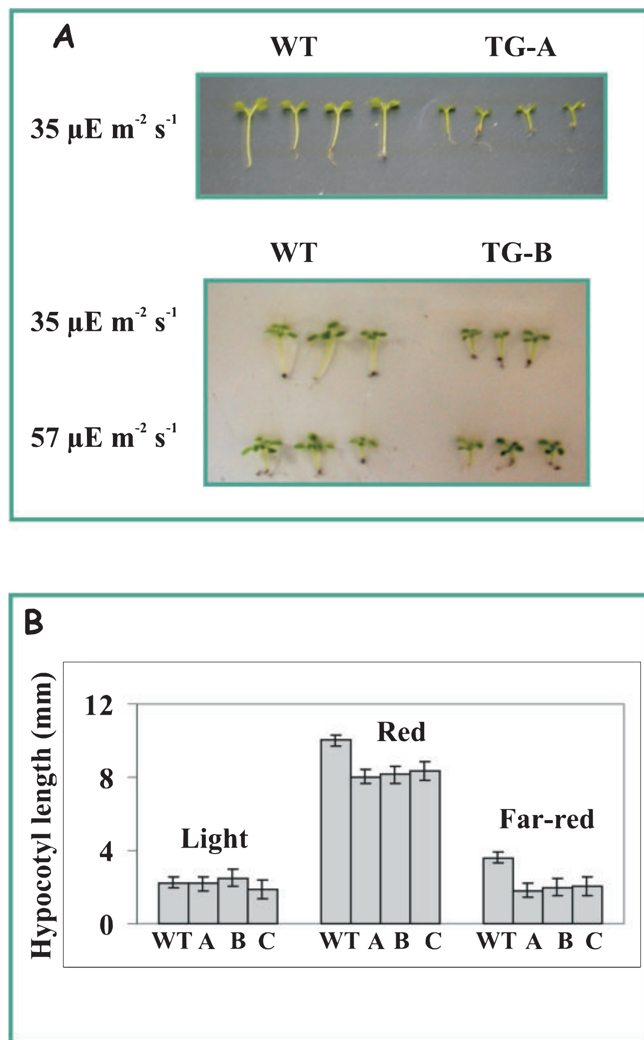

Fig. 5 *35S:Hahb-10* transgenic plants are less affected by changes in illumination intensity than their wild-type counterparts. (A) Comparison between transformed and control 7-day-old seedlings grown at 35 μE m^{-2} s^{-1} (upper panel) and between 14-day-old plants grown at 57 or 35 μE m^{-2} s^{-1} as indicated (lower panel). (B) Hypocotyl length in 5-day-old seedlings grown on soil with different light qualities. This is a representative experiment done with plants of each genotype (n = 20). Controls were done with normal illumination conditions as described in Materials and Methods. WT, wild-type seedlings; A, transgenic seedlings line A; B, transgenic seedlings line B; C, transgenic seedlings line C. Red- and far-red-enriched illumination was obtained as described in Materials and Methods.

development. Table 3 illustrates experiments performed with the three independent *Hahb-10*-overexpressing lines and wild-type plants grown under normal illumination conditions as described in Materials and Methods. Stem height was greater in all transgenic lines until 35 d after germination. This difference disappeared at day 45, showing both plant types the same height but not the same maturation grade. Seed maturation began approximately 50 d after germination in transgenic

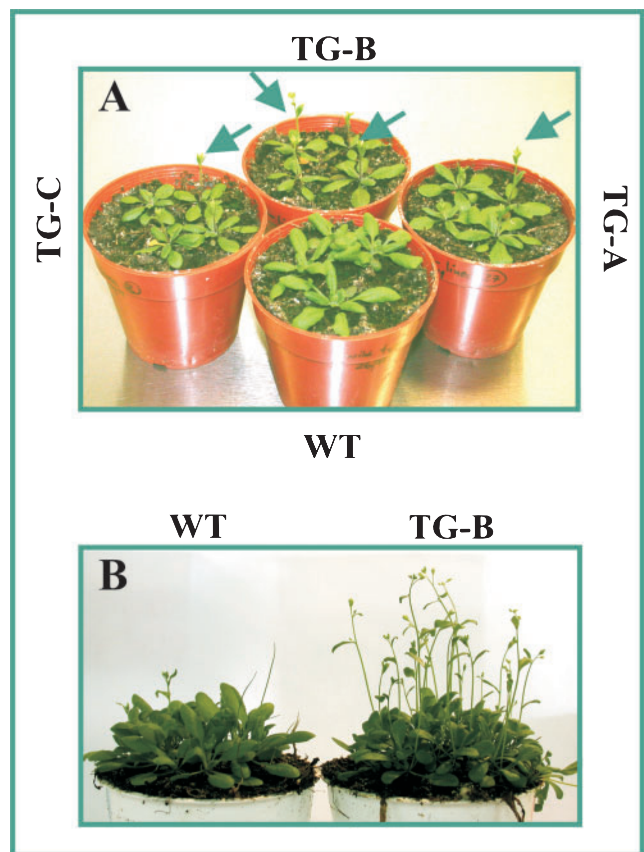

Fig. 6 *35S:Hahb-10* transgenic plants develop faster than non-transformed plants. (A) Comparison of developmental state between transformed and control 21-day-old plants grown on soil (four plants per 8 cm diameter pot). (B) Difference in the developmental state between transgenic and non-transformed plants in a typical experiment done with 20 plants per 8 cm diameter pot.

Table 3 *Hahb-10* transgenic plant stems elongate faster than those of non-transformed plants

Plant age (d)	WT plants	TG line A	TG line B	TG line C
28	13.82 ± 1.01	19.45 ± 1.71	18.32 ± 3.1	18.44 ± 1.05
35	27.00 ± 3.79	30.00 ± 1.17	31.06 ± 2.75	28.76 ± 1.79
45	31.20 ± 2.00	31.62 ± 1.04	32.67 ± 1.60	29.2 ± 1.28

Stem height of *Hahb-10*-expressing transgenic and non-transformed Arabidopsis plants was measured with a ruler at different times after sowing. This is a representative experiment done with 20 plants of each genotype. Stem length is expressed in mm.

plants while, under the same conditions, wild-type plants started this process approximately 65 d after germination. Collection of seeds could be done 2 weeks in advance in the *Hahb-10*-overexpressing genotype compared with the wild-type. Seed production was similar in transgenic and non-transformed plants when the number of plants per pot was either one or four.

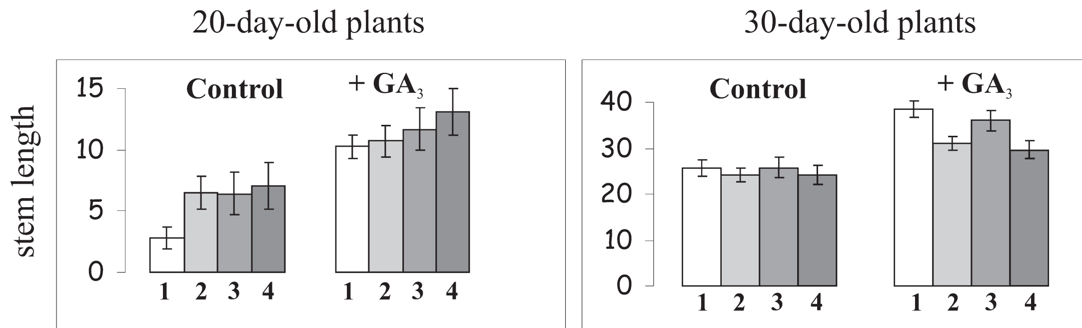

Fig. 7 Stem elongation is almost unaffected in gibberellin-treated transgenic plants. Stem height was measured with a ruler in non-transformed (1) or *35S-Hahb-10* transgenic plants (2, line -A; 3, line -B; and 4, line -C). Treatment with GA_3 is represented in the right hand series of each panel. The first panel represents the observed results in 20-day-old plants while the second panel shows the results observed after a second hormone treatment in 30-day-old plants.

Significant differences were observed between experiments performed with either two, five or 20 plants per pot. When the number of plants per pot increased, the difference in developmental rate between transgenic and non-transformed individuals increased, i.e. under space-, light- and/or nutrient-limiting conditions, *Hahb-10*-overexpressing plants show a strong response to shade avoidance, retaining their developmental rate, while their non-transformed counterparts decreased in growth rate (Fig. 6, lower panel).

Effect of gibberellins on Hahb-10-overexpressing plants

Among the hormones tested as effectors of sunflower *Hahb-10* expression, gibberellins proved to be positive regulators. In order to investigate the response of *Hahb-10*-overexpressing lines to this hormone, we treated plants with 200 μM gibberellins as described in Materials and Methods. As can be observed in Fig. 7, non-transformed plants showed a 3.7-fold increase in stem elongation with respect to their non-treated controls 5 d after the treatment, while transgenic stem length increased only 1.6-fold. Treated wild-type plants had only seven developed rosette leaves when the inflorescence primordium appeared, while transgenic plants flowered at the same stage (seven rosette leaves) whether they were treated or not. After the second treatment, a similar result was observed, i.e. transgenic plants were almost insensitive to the hormone treatment (1.2-fold induction in average) compared with wild-type plants (1.5-fold induction in average), indicating that *Hahb-10*-overexpressing plants show characteristics of gibberellin-treated non-transformed plants.

Possible target genes of Hahb-10

In order to investigate the action mechanism of *Hahb-10*, we have also analysed the expression levels of several genes that are regulated by illumination conditions (Dae-Shilk et al. 2003). We have prepared probes for *PsbS* (At1g44575), *CAB2* (At1g29920) and *CHS* (At5g13930), encoding a PSII-associated protein, a Chl a/b-binding protein and chalcone synthase, respectively. Notably, steady-state transcript levels of *PsbS* showed significant changes in *Hahb-10*-overexpressing plants with respect to control plants, especially under a normal illumination regime (Fig. 8). This gene diminishes its expression level about 4- to 5-fold under normal illumination in transgenic plants compared with the gene expression level in non-transformed plants under the same conditions. Similar results were observed when etiolated seedlings were transferred to light for 8 h, i.e. *PsbS* shows a lower expression level in transgenic plants after the treatment, and trancripts were almost

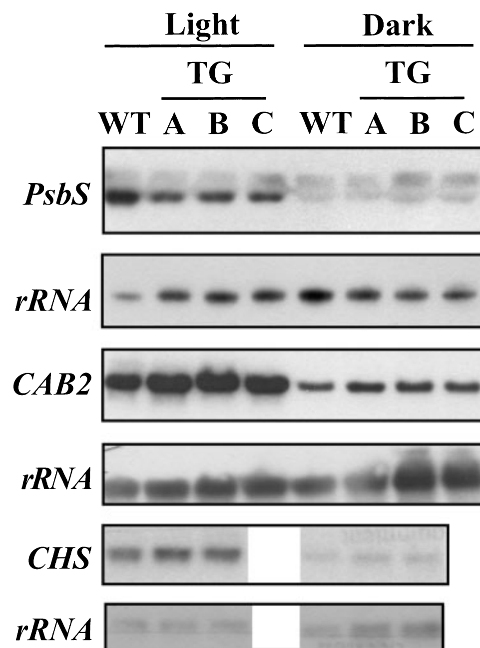

Fig. 8 Expression of the *PsbS* gene is reduced in *Hahb-10*-overexpressing transgenic plants. Northern blot analysis of non-transformed and transgenic Arabidopsis plants. Total RNA (10 μg) was extracted from wild-type (WT) or transgenic seedlings (line -A, -B or -C) overexpressing *Hahb-10* grown under normal illumination conditions during 4 d. Light, plants were in the light for an additional 8 h; dark, plants were in the dark for an additional 8 h. Probes specific for *PsbS*, *CAB2*, *CHS* or rRNA were used.

undetectable in etiolated seedlings in both genotypes. Neither *CAB2* nor *CHS* showed significant differences in their expression levels when comparing transgenic and wild-type plants (Fig. 8). In the dark, very low and similar expression levels were observed in all genotypes for the three genes analysed. Since the in vitro sequence bound by Hahb-10 [CAAT(C/G)ATTG (Tron et al. 2002)] is not present in the *PsbS* promoter region (3,000 bp upstream of the transcription initiation site), *PsbS* may be an indirect target gene of the transcription factor Hahb-10. Hahb-10 may affect signal transduction pathway(s) that are involved in regulating the response of *PsbS* to different light conditions.

Discussion

Hahb-10 has been shown to encode an HD-Zip transcription factor that belongs to the sunflower HD-Zip II subfamily (Gonzalez et al. 1997, Tron et al. 2002). We describe here that expression of this gene is rather high in photosynthetic tissues, especially in leaves. The induction of *Hahb-10* expression in etiolated seedlings suggests that it may have a role in signalling illumination conditions. To address this question, we used an overexpression approach choosing *Arabidopsis* as a model system. The expression levels of *Hahb-10* in the *35S:Hahb-10* transgenic lines used in our study are higher with respect to their *Arabidopsis* counterparts under control conditions and to its own expression level in sunflower.

Vegetative to reproductive stage transition is a complex process. Flowering is controlled by four major pathways: photoperiod, gibberellins, vernalization and an autonomous pathway. This transition in transgenic plants occurs earlier than in non-transformed plants. An important group of plants that includes *Arabidopsis* requires appropriate environmental conditions to develop flowers (Cerdan and Chory 2003). Light and temperature are two factors that profoundly affect flowering time. It seems that *Hahb-10* somehow influences signal transduction pathways involved in flowering. The pronounced effect observed on growth when the density of plants increased may also reflect a better response of the transgenic plants to shading, supporting that *Hahb-10* is involved in perceiving the red/far-red light ratio. Additional experiments must be performed to elucidate the mechanism involved.

The observations made with *Hahb-10* resemble in part those made with *Athb-2/HAT4*. Similarities include the induction of both genes in dark-adapted plants and the high steady-state transcript levels detected in the vegetative phase of plant growth. Plants that overproduce either of these genes are darker green than wild-type plants, have smaller roots, less expanded cotyledons and a shorter life cycle. On the other hand, some clear differences exist: *HAT4/Athb-2* increases its transcript levels in flowering plants while *Hahb-10* does not. Overproduction of *HAT4/Athb-2* results in a germination defect in the dark, while an opposite effect is observed with the sunflower gene, i.e. *Hahb-10*-overexpressing plants germinate faster than the wild-type and became healthier after long periods of etiolation. In addition, *HAT4/Athb-2*-overexpressing plants present longer hypocotyls (Schena et al. 1993, Steindler et al. 1999) while *Hahb-10* transgenic plants present shorter ones with respect to non-transformed plants. These observations led us to conclude that these genes are not orthologues and that they have different functions in both plant species, although they have a conserved HD-Zip domain sequence.

Athb-2/HAT4 has been described as a negative regulator of paralogous genes. It recognizes its own promoter region and its endogenous expression is repressed in overexpressing transgenic plants (Steindler et al. 1999, Ohgishi et al. 2001). The observations made in *Hahb-10* transgenic plants cannot be explained as an effect of the repression of Arabidopsis homologues. Indeed, *HAT4/Athb-2* and *HAT22* transcript levels do not change in *Hahb-10*-overexpressing plants.

Gibberellins are a group of hormones involved in a wide variety of developmental processes including stem elongation and various aspects of seed germination. In reproductive development, gibberellins can affect the transition from the juvenile to the mature stage, as well as floral initiation, sex determination and fruit set (Taiz and Zeiger 1998). Plants overexpressing *GA-20 oxidase*, a gene involved in gibberellin biosynthesis, flower earlier than their non-transformed counterparts (Huang et al. 1998). In the present study, it was observed that GA_3 induces *Hahb-10* expression in sunflower and that overexpressing plants show some characteristics of plants that have previously been treated with gibberellins. This fact allows us to speculate that *Hahb-10* may be a positive regulator of gibberellin biosynthesis and/or action. This conclusion is based on three observations: seed germination, accelerated transition from juvenile to the adult phase and earlier floral initiation. These phenomena are affected by gibberellins and are also affected in *Hahb-10*-overexpressing plants. The behaviour of the *Hahb-10* gene when plants are treated with gibberellins resembles that of *Arabidopsis HAT2* when plants are treated with auxins (Morelli and Ruberti 2002). In both cases, HD-Zip gene expression is induced by a hormone, and overexpressing plants are almost insensitive to treatment with the same hormone.

Phytochromes are photochromic proteins that regulate light responses under different light conditions (quantity, quality and timing). *Arabidopsis thaliana* has five phytochrome-encoding genes (*PHYA–PHYE*). *PHYA* and *PHYB* are involved in regulating flowering time in antagonistic forms. Transgenic plants overexpressing *PHYA* present an earlier flowering time compared with non-transformed plants (Bagnall et al. 1995). Phytochrome B is involved in detecting red to far-red light ratios associated with plant density (Casal et al. 2004). Transgenic potato plants that ectopically express the *Arabidopsis PHYB* gene show increased tuber yield when grown in the field. It has been reported that this effect is larger at very high densities (Boccalandro et al. 2003). We have observed and described here that *Hahb-10*-overexpressing plants present a shortened flowering time, and this effect is also more pro-

nounced at high densities. We have then observed in our transgenic plants some similar phenotype to those exhibited by *PHYA*- or *PHYB*-overexpressing plants. It is possible then that the sunflower gene is implicated in subsets of signal transduction pathways regulated by different phytochromes.

A good number of genes are regulated by phytochromes, constituting the molecular basis for the phytochrome developmental changes under far-red/red light (Ma et al. 2001). According to the effects produced by light in *Hahb-10* expression in sunflower and the behaviour of *Hahb-10*-overexpressing plants, we have decided to analyse some of the expression of known phytochrome-dependent genes. We have examined the transcript levels of three light-inducible genes (*CAB*, *PsbS* and *CHS*) and found a strong decrease in light induction of *PsbS* expression in transgenic plants. This finding may indicate that *Hahb-10* directly or indirectly negatively regulates *PsbS* expression, especially under normal illumination conditions. Since Arabidopsis *PsbS* does not have the sequence bound in vitro by Hahb-10 and other members of the HD-Zip II subfamily (Tron et al. 2002), it is reasonable to think that it is an indirect target. On the other hand, neither *CHS* nor *CAB2* showed a differential behaviour between transgenic and non-transformed plants, indicating that they are probably affected by other signalling pathways. Using a very different strategy, other authors also suggested different regulatory mechanisms for these light-inducible genes (Dae-Shilk et al. 2003).

In summary, *Hahb-10* seems to be involved in modulating several plant responses to light conditions. Considering the complexity of these responses, future efforts will be devoted to analyse the transduction pathways that are affected in *Hahb-10*-overexpressing plants.

Great efforts to reduce the life cycle have been made by breeding and genetic engineering. The success was limited owing to the genetic complexity of the responses. The improved understanding of the role of novel genes in adaptation to environmental conditions will provide the basis of effective engineering strategies leading to better adapted plants. The results reported here allow us to envisage a biotechnological use of *Hahb-10* for the reduction of the life cycle and for increasing planting densities.

Materials and Methods

Plant material, growth conditions and treatments

Helianthus annuus L. (sunflower cv. contiflor 15, from Zeneca, Balcarce, Argentina) seeds were surface sterilized and grown on filter paper inside Petri dishes during 4 d in the dark or under different illumination conditions as described in the figure legends. Seedlings were then transferred to plastic supports containing soil and grown for variable times depending on the purpose of the experiment. Water stress was imposed by transferring 7-day-old seedlings to Petri dishes with dry filter paper. Cold shock was imposed by transferring 7-day-old seedlings grown in Petri dishes to a cold chamber maintained at 4°C during 4 h. Heat shock was imposed in a similar way, but plants were transferred to a 45°C regulated incubator. Hormone treatments were carried out by transferring 7-day-old seedlings to Petri dishes with fresh MS medium/Gamborg vitamins supplemented with either 200 μM gibberellin (GA_3 for plant tissue culture, Sigma, St. Louis, MO, USA), 10 μM ABA or sprayed with Ethrel*, an ethylene-releasing agent (2-chloroethylphosphoric acid).

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ, USA). Plants were grown on soil in a growth chamber at 22–24°C under long-day photoperiods (16 h of illumination by a mixture of cool-white and GroLux fluorescent lamps) at an intensity of 100 $\mu E\ m^{-2}\ s^{-1}$. These conditions are named 'normal growth conditions' throughout the text. Plants used for the different treatments or for transformation were grown in 8 cm diameter ×7 cm height pots during the times indicated in the figures.

Transformation and identification of transformed plants

Transformed *Agrobacterium tumefaciens* strain GV2260 was used to obtain transgenic *Arabidopsis* plants by the floral dip procedure (Clough and Bent 1998). Transformed plants were selected on the basis of kanamycin resistance and positive PCR, carried out on genomic DNA with the specific oligonucleotides H1 (5′-CggTggT-TCgTCgTTCT-3′) and H2 (5′-CCgAATTCCCgATCTgTTCACAC-gAC-3′). To assess *Hahb-10* expression, Northern blot analysis was performed on T_2 transformants. Three positive independent lines (arising from two different transformation experiments) were further reproduced, and homozygous T_3 and T_4 plants were used to analyse the expression levels of *Hahb-10* and the phenotype of transgenic plants. Plants transformed with pBI101.3 or pBI121 were used as controls.

Treatment of transgenic plants with variations in the illumination conditions

Control conditions of growth have been defined above (16 h of illumination by a mixture of cool-white and GroLux fluorescent lamps at an intensity of 100 $\mu E\ m^{-2}\ s^{-1}$). Some experiments, as detailed in the figures legends, were carried out with the same photoperiod but at an intensity of 57 or 35 $\mu E\ m^{-2}\ s^{-1}$ achieved by changing the distance from the light source.

Far-red-enriched light was obtained using a set of incandescent lamps, and a combination of 3 mm red and blue acrylic filters with a water refrigerating interface in a closed chamber isolated from other light sources inside a temperature-regulated culture room. Filtered light under these conditions irradiates at a wavelength between 680 and 700 nm as measured with a Shimadzu UV-VIS spectrophotometer. Red-enriched light was obtained in the same form, using red and yellow acrylic filters.

Experiments to detect possible target genes were done in the following way: 4-day-old seedlings germinated and grown under a normal photoperiod as described were then subjected to 8 h of total darkness (light/dark) or kept under the same conditions for the same period of time (light). After this treatment, plants were immediately collected and total RNA from the different samples was extracted as described above.

Chlorophyll and anthocyanin measurements

For pigment content in transgenic and wild-type plants, 200 mg of 21-day-old leaves of each genotype were collected and frozen with liquid nitrogen. A 10 ml aliquot of 1% (w/v) HCl in methanol was added and the mixture was incubated overnight at 4°C. Anthocyanin concentration was determined in filtered extracts according to Mancinelli (1990). Chl concentration was determined according to the method described by Whatley and Arnon (1963) on samples obtained from 200 mg of leaves and incubated overnight with absolute ethanol in the dark.

Treatment of transgenic plants with gibberellins

Arabidopsis transgenic or wild-type plants were grown under the conditions described above during 15 d. After this, the plants were sprayed with 200 μM GA_3. This treatment was repeated at day 25. Stem length was measured in 20- and 30-day-old plants with a ruler.

Northern analysis

Total RNA from sunflower or from *Arabidopsis* was isolated and prepared as described by Almoguera and Jordano (1992) or Carpenter and Simon (1998). For Northern analysis, RNA was denatured with formamide and formaldehyde, separated in a 1.5% (w/v) agarose/6% formaldehyde gel, and blotted onto nylon membranes (Hybond N, Amersham Biosciences, Buckinghamshire, UK) essentially as described by Sambrook et al. (1989). Before blotting, a UV image was taken to ensure similar loading of the different analysed samples in the agarose gel. Hybridization was performed overnight at 65°C in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0), 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) Ficoll, 0.5% (w/v) SDS. An *Eco*RI/*Spe*I fragment (from +1 to +275), corresponding to the 5′-non-coding region of the *Hahb-10* cDNA plus the first 241 nucleotides of the coding region, which does not include the HD-Zip domain, was labelled with [^{32}P]dATP (1×10^8 dpm μg^{-1}) by random priming (Sambrook et al. 1989) and used as probe. Filters were autoradiographed using Bio-Max films and transcreen (Kodak) overnight. To check the amount of total RNA loaded and transferred in each lane, filters were then re-probed with a 25S rRNA probe from *Vicia faba* under similar conditions to those described above, except that hybridization was performed at 62°C.

Probes for the *PsbS*, *CAB2* and *CHS* genes were obtained from clones APZ10g10, APZ49a09 and RZ115f04, respectively, kindly provided by the Kazusa DNA Research Institute (Japan). Hybridization was done under similar conditions to those described for the *Hahb-10* probe.

Spots obtained with the specific probe were quantitated and referred to their rRNA using Image Pro Plus software.

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