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ORIGINAL ARTICLE

The intron of the *Arabidopsis thaliana COX5*c gene is able to improve the drought tolerance conferred by the sunflower *Hahb*-4 transcription factor

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Abstract *Hahb*-4 is a member of *Helianthus annuus* (sunflower) subfamily I of HD-Zip proteins. Transgenic *Arabidopsis thaliana* plants constitutively expressing this gene exhibit a strong tolerance of water stress in concert with morphological defects and a delay in development. In order to obtain a drought-tolerant phenotype without morphological associated phenotype, several stress inducible promoters were isolated and transgenic plants expressing *Hahb*-4 controlled by them were obtained and analyzed. These plants showed unchanged morphology in normal growth conditions and enhanced drought tolerance compared with non-transformed plants, but no as high as the one exhibited by the constitutively transformed genotype. A chimerical construction between the *Hahb*-4 promoter and the leader intron of the Arabidopsis *Cox5*c gene was made either directing *gus* or *Hahb*-4 expression. GUS activity increased in transgenic plants after induction, showing the same distribution pattern as in plants transformed with a construction lacking the intron. Transgenic plants, bearing the chimerical construct, are indistinguishable from wild type plants in normal growth conditions whereas the water stress tolerance achieved was as strong as the one shown by the constitutive genotype. This enhanced stress tolerance seemed to be due to a combination of an increase in transcription and translation rates in comparison to those of plants transformed with the *Hahb*-4 promoter. Similar strategies could be applied in the future for the obtaining of suitable promoters responsive to other external agents.

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Keywords *COX5*c (gene encoding cytochrome c oxidase subunit) · *Hahb*-4 · HD-Zip protein · Intron · Promoter · Tolerance (drought)

Abbreviations

ABA	Abscisic acid
IME	Intron-mediated enhancement
LPF	*Hahb*-4 large promoter fragment
SPF	*Hahb*-4 short promoter fragment

Introduction

Great efforts were devoted using plant breeding methods in order to improve tolerance of drought, salinity and other abiotic stresses. Advances in Genetic Engineering have enabled us to introduce a particular gene in a plant to confer on it a desired phenotype, In order to achieve this aim, it is necessary to identify the gene able to confer the desired characteristic and a promoter able to drive its expression in an accurate time and space pattern. Constitutive promoters are useful to determine genes function, but generally produce unnecessary metabolic expenses in transgenic genotypes.

Among the genes involved in water deficit stress responses, transcription factors are thought to be "master switches". Up to now, various transcription factors from several families were shown to be related to these responses (Jaglo-Ottosen et al. 1998; Liu et al. 1998, 1999; Kawasaki et al. 2001; Seki et al. 2002; Bray 2004; Maruyama et al. 2004). Most of them act as direct or indirect regulators of drought-responsive genes expression.

HD-Zip proteins, unique to plants, were proposed as good candidates to trigger developmental responses to environmental conditions, a characteristic feature of plants

(Schena and Davies 1993; Chan et al. 1998). Some authors informed that expression of members of the HD-Zip family of transcription factors is regulated by external factors like illumination or water stress (Schena and Davis 1992; Carabelli et al. 1993; Schena et al. 1993; Söderman et al. 1994, 1996, 1999; Frank et al. 1998; Lee and Chun 1998; Deng et al. 2002, 2006; Rueda et al. 2005).

Hahb-4 is a transcription factor that belongs to the sunflower HD-Zip I subfamily (Gago et al. 2002). This protein confers drought tolerance on transformed *Arabidopsis* plants either when it is constitutively expressed or when its expression is driven by its own water stress inducible promoter (Dezar et al. 2005a; Manavella et al. 2006). This tolerance occurs concomitantly with a delay in ethylene-mediated senescence (Manavella et al. 2006). However, when this gene was highly constitutively expressed water-stress tolerance was associated with undesirable morphological characteristics, such as a marked delay in development, whereas expression controlled by its own promoter (*LPF:Hahb*-4) resulted in plants indistinguishable from wild type plants but exhibiting a weaker stress tolerance. Transcriptome analysis of *Hahb*-4 expressing transgenic plants revealed that a large number of target genes is regulated similarly in both the inducible and the constitutive genotypes (Manavella et al. 2006). Therefore, we wondered why there are so many phenotypic differences between these two types of plants. Transcript levels in *LPF:Hahb*4 plants in control conditions are low but detectable by qRT-PCR and they significantly increase when plants are subjected to severe water stress, ethylene or ABA treatments (Dezar et al. 2005b). Even so, the rate of induction or the final achieved level was not enough to confer to transgenic plants a tolerance as high as when the gene was constitutively expressed.

We intended to design a strategy to obtain stress tolerant transgenic plants without morphological changes. In this paper we describe the obtaining of transgenic plants with several constructs bearing the *Hahb*-4 cDNA controlled by inducible promoters. All of them show similar results to those of the *Hahb*-4 promoter, resulting in transgenic plants with normal morphological phenotypes and an intermediate drought tolerance.

An enhancement of expression by introns was reported for several cases from monocot (Callis et al. 1987; McElroy et al. 1990; Christensen et al. 1992; Xu et al. 1994; Jeon et al. 2000; Morello et al. 2002) and dicot plants (Norris et al. 1993; Gidekel et al. 1996; Rose and Last 1997; Plesse et al. 2001; Mun et al. 2002). Introns that influence expression are more frequently located near the translation start site within non-coding regions. The exact role of introns in promoting an increase in expression levels remains unclear. Some introns seem to contain transcriptionally active regulatory elements (Gidekel et al. 1996), while others apparently act post-transcriptionally (Rose and Last 1997), suggesting the existence of diverse mechanisms of action. Mutation of 5′ and 3′ splice sites in the PAT1 intron prevented splicing as well as when U-richness is reduced (Rose 2002). In the same way, enhancement was abolished when simultaneously eliminating branschpoints and the 5′ splice site, structures involved in the first two steps of the spliceosome assembly indicating that IME (intron-mediated enhancement) absolutely requires either of these sequences, even though IME is not eliminated when each is individually mutated. On the other hand, 3′ splice site of this intron contributes to but is not essential for IME (Rose 2002).

It has recently been proposed that many introns would act by increasing the processivity of the transcription machinery (Rose 2004). *Arabidopsis thaliana* has three nuclear genes encoding subunit 5c of mitochondrial cytochrome *c* oxidase (*COX5c)*. Their non-coding regions include a leader intron inserted in the 5′ non-coding segment (Curi et al. 2005). Removal of the leader intron produced a significant decrease in expression to values only slightly higher than those observed with a promoterless *gus* gene. However, a construct that only has the intron directing *gus* expression is absolutely inactive (G.C. Curi, Natl. Univ. Litoral, Santa Fe, Argentina, personal communication), indicating that this intron could enhance the expression driven by other *cis*-acting elements present in the promoter but it is not autonomous to drive expression by itself. Moreover, the intron increased GUS expression levels only when fused in the correct orientation with the promoter of the related *COX5b-1* gene. Comparison of GUS activity values with the transcript levels suggests that it also increases translation efficiency of the corresponding mRNA (Curi et al. 2005).

In this paper we describe the construction of a chimera bearing the sunflower *Hahb*-4 promoter fused to the leader intron of the *Arabidopsis thaliana COX5*c gene driving the expression of either the *gus* reporter or the *Hahb*-4 cDNA. Plants transformed with these constructs are, by visual inspection, undistinguishable from non-transformed plants but they show an enhanced *gus* expression pattern and a water stress tolerance as high as the one shown by plants that express *Hahb*-4 constitutively. We propose that this chimera is suitable for the expression in transgenic plants of genes whose function is needed only under stress conditions.

Materials and methods

Plant material and growth conditions

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 with a mixture of cool-white and GroLux fluorescent lamps) at an intensity of approximately 150 µmol m^{-2} s^{-1}. Plants used for the different treatments were grown in 8 cm diameter × 7 cm height pots for the time periods indicated in the figure legends.

Water stress treatments

Water stress treatments in soil were carried out as follows: 1 l of water was added to a 35-cm plastic square tray. Sixteen 8 × 9 cm pots, each with 130 g of soil, were placed in the tray. Four seeds were sowed in each pot and the trays were transferred to culture conditions as described above and watered during the first 20 days of development. When severe damage of non-transformed plants was observed, 14 days after initiating the treatment, plants were re-watered. Samples for RNA or GUS analysis were extracted from plants collected at different times as described in the figure legends. Survivor plants were counted and analyzed after re-watering.

Genomic DNA isolation

Isolation of soybean and *Arabidopsis* promoters was performed by PCR on genomic DNA extracted according to Doyle and Doyle (1987). Specific oligonucleotides were designed as follows:

rd29: rd29F: 5′-CGCAAGCTTCGATAGGGAAGTGATGTAGG -3′; rd29R: 5′- CCGGGATCCACTCTTTGTGTGACTGAGG- 3′

AtS1: ats1F: 5′-CGCAAGCTTGGTGAATTAAGAGGAGAGAG -3′; ats1R: 5′- GGCGGATCCTCCAATAGAAGTAATCAAACC -3′

sLEA: sleaF: : 5′-GCCGTCGACGCGCACACCAACTTACAAC -3′; sleaR: 5′- CGCGGATCCCTCACTTAGGTTCTTTCTTCT- 3′

Constructs

In the constructs described below, only the coding region of *Hahb*-4 (from start to stop codons inclusive) was inserted between the promoter and the *nos* termination sequence with an extra ACC triplet just before the ATG to improve translation efficiency (Fütterer and Hohn 1996). *E. coli* DH5α cells were transformed with each construct and, once positive clones were obtained and sequenced, *Agrobacterium tumefaciens* cells were transformed (Höfgen and Willmitzer 1988) with the same plasmid.

Constructs *Ats1A:Hahb4, rd29:Hahb4* and *GmPM9:Hahb4*

Ats1A, *rd29 and GmPM9* promoters (Accession No. NM_202369, NM_124610 and M97285, respectively) corresponding to the small subunit of *RubisCO*, *rd29* and soybean LEA (Late Embryogenesis Abundant protein) encoding genes respectively were cloned in the *Sal*I/*Bam*HI sites of a previously obtained construct bearing the *Hahb*-4 cDNA in pBI121 (Dezar et al. 2005a) after digestion with the same enzymes in order to delete the 35S CaMV constitutive promoter.

Constructs *SPF/LPF:Hahb4*

These constructs in pBI101.3 were obtained as previously described (Manavella et al. 2006).

Constructs *SPF/LPF:Intron:gus*

The *SPF/LPF* segments (1,015 and 1,225 bp, respectively) were isolated from a previously obtained T-easy clone (Dezar et al. 2005b) by digestion with *Sal*I/*Bam*HI and cloned in a pBluescritpt SK^{-} using the same restriction sites. These new clones, named pBSKSPF and pBSKLPF, were digested with *Sal*I/*Xba*I and cloned in pBI101.3 using the same restriction sites. The resulting clones were termed pBLPF/*gus* and pBSLPF/*gus*. The leader intron of *Cox5*c-2, previously cloned into the TOPO plasmid (Curi et al. 2005) was isolated by restriction with *Xba*I and cloned in the unique *Xba*I site of pBSLPF/*gus*. The orientation of the insertion was checked by PCR using specific oligonucleotides. The resulting constructs were named *SPF:I:gus* and *LPF:I:gus*.

Constructs *SPF/LPF:I:Hahb4*

The novel clones (see below), pBSKSPF and pBSKLPF, were digested with *Sal*I/*Bam*HI whereas the *Hahb*-4 cDNA was cloned into the *Bam*HI/*Sac*I sites of the pBI101.3 after restriction with the same enzymes of a previously obtained clone (Dezar et al. 2005a, b). This new clone was named pBIHahb4. LPF and SPF (digested with *Sal*I/*Bam*HI) were introduced into the same sites in this pBIHahb4 resulting in the clones pBISHahb4 and pBILHahb4. Finally, the leader intron of *Arabidopsis Cox5*c-2 was inserted in pBISHahb4 and pBILHahb4 in their unique *Xba*I sites. The resulting constructs were termed *SPF:I:Hahb4* and *LPF:I:Hahb4*. The orientation of the insertion was checked by PCR using specific oligonucleotides.

Transformation and identification of transformed plants

Transformed *Agrobacterium tumefaciens* strain LBA4404 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 spe-

cific oligonucleotides for each construct described above. Three/four positive independent lines (arising from two transformation experiments) were further reproduced and homozygous T3 and T4 plants were used in order to analyze the expression levels of *Hahb*-4 and the phenotype of transgenic plants. Plants transformed with pBI101.3 or pBI121, used as negative or positive controls, respectively, or with a construction bearing the *Hahb*-4 promoter region fused to the reporter gene *gus*, were obtained in a similar way. In all cases three/four independent homozygous transformed lines (T3 and/or T4) were analyzed.

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-β-glucuronic acid solution in 100 mM sodium phosphate pH 7.0 and 0.1% Triton X-100 and, after applying vacuum for 5 min, they were incubated at 37°C overnight. Chlorophyll was cleared from the plant tissues by immersing them in 70% ethanol.

Real time RT-PCR measurements

RNA for real-time RT-PCR was prepared with Trizol® reagent (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer´s instructions. RNA (2 µg) was used for the RT reactions using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative PCRs were carried out using a MJ-Cromos 4 apparatus in 25 µl final volume containing 1 µl SyBr green (10×), 10 pmol of each primer, 3 mM $MgCl_2$, 5 µl of the RT reaction and 0.15 µl Platinum Taq (Invitrogen). Fluorescence was measured at 80–84°C for 40 cycles. Transcript levels were normalized using actin as control as previously described (Charrier et al. 2002).

Specific oligonucleotides for *Hahb*-4 or *gus* genes were designed. Their sequences were:

Hahb4R: 5′-GATTCTTCACCGCTGCCACTACT-3′; Hahb4F: 5′-AACGCGCTAAAGCATAACTACGAG-3′; gusR: 5′-AAGCCGACAGCAGCACTTTCATC-3′; gusF: 5′-TGCGGACTTACGTGGCAAAGGAT-3′.

T-DNA insertions were quantified by real time PCR using as template 60 ng of purified genomic DNA extracted from each transgenic line and the oligonucleotides Hahb4R and Hahb4F (see above). The insertion numbers were determined using the transgenic lines *35S:Hahb4*-A and –B (one and two copies, respectively) as standard which were previously analyzed by Southern blot (Dezar et al 2005a).

Transpiration analysis

Transpiration rate and stomatal conductance measurements were carried out with detached leaves using an open gas-exchange system with infrared gas analyzer (IRGA, Qubit Systems Inc., Kingston, On., Canada). A minimum of three expanded leaves were sealed into the chamber illuminated with PPF of 150 µmol m^{-2} s^{-1}. The temperature was maintained between 24 and 26°C. Room humidity was used in air flows with an average Relative Humidity of 50, 5%. Measurements were taken at least three times for each independent transgenic or wild type plant using four different individuals of each. The leaves surface area was calculated by scanning.

Results

Promoters from *Arabidopsis thaliana rd29*, soybean *GmPM9* and sunflower *Hahb*-4 driving *Hahb*-4 expression produce indistinguishable phenotypes in transgenic plants

Constructs bearing the promoters of *rd29*, *Ats1A* (RubisCO small subunit) from *Arabidopsis thaliana* and *GmPM9* from soybean, controlling the expression of *Hahb*-4 were obtained. *Arabidopsis* plants were transformed with these constructs and three independent transgenic homozygous lines from the third generation (T3) of each genotype were analyzed and compared with the previously obtained *35S:Hahb4* and *LPF/SPF:Hahb4* plants. Morphological characteristics including leaves shape, color and form, stem length and root tail were almost identical for *rd29:Hahb4*, *SPF/LPF:Hahb4* and *GmPM9:Hahb4* genotypes when grown in normal culture conditions, whereas the *Ats1A:Hahb4* genotype shows some phenotypical features similar to those exhibited by the *35S:Hahb4* plants. In these plants, leaves are more rounded and stems are shorter than those of the other transgenic genotypes. Figure 1 and Table 1 show photographs and morphological parameters of all these transgenic plants in the same state of development as well as *35S:gus* plants used as reference. Transpiration rates as well as stomatal conductance of all the genotypes were quantified indicating that no significant differences in these parameters are exhibited in normal growth conditions (Table 1, last columns). The number of T-DNA insertions was quantified for each line by real time PCR and vary from 1 to 5 in accordance with the values reported by other authors (Radchuk 2005), suggesting that morphological characteristics and/or expression levels are independent from this number. This fact indicates that this transgene expression level, as in other cases, does not directly depend on the number of insertions. Actually, it may be the result of the combination of a set of complex factors.

All the novel transgenic genotypes show a similar water stress tolerance

All the novel transgenic genotypes tolerated the water stress treatment to which they were subjected more

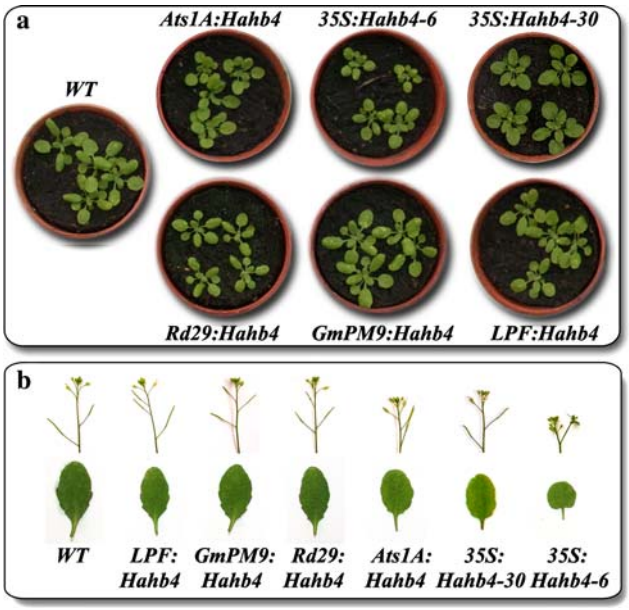

Fig. 1 Phenotype of transgenic plants expressing *Hahb*-4 by the control of different promoters. **a** Photographs of 20-day-old plants grown on soil belonging to different genotypes. **b** Details of leaves and inflorescence morphologies. Leaves were cut from 35-day-old plants whereas inflorescences are from 45-day-old plants. High-expressing *35S:Hahb4* is represented by line 6 (*35S:Hahb4*-6) and low-expressing *35S:Hahb4* by line 30 (*35S:Hahb4*-30)

efficiently than their wild type counterparts, although their tolerance is clearly more limited than the one showed by the constitutively expressing plants. This could be indicating that the initial trancript level or/and the rate or level of induction by drought is not enough to switch on an optimal response to this type of stress. Table 2 shows typical experiments where transgenic plants were subjected to severe water stress as described in the Materials and methods, after what they were irrigated and counted. Considering the data displayed in Table 2, we can conclude that neither the *SPF/LPF*, nor the *rd29*, *GmPM9* and *Ats1A* promoters are as effective as the 35S CaMV in driving the expression of *Hahb*-4 so as to confer stress tolerance. Concomitantly, stomatal conductance and transpiration rates of these genotypes are more similar to those shown by WT plants than to those of the constitutive genotype. It is also certain that the expression driven by these promoters is in every case too low or slow to cause the undesirable morphological characteristics observed in the constitutive genotype. The exception was *Ats1A* which generated some morphological alterations, although not very pronounced.

The leader intron of *Arabidopsis thaliana Cox5*c enhanced *gus* expression when fused to the sunflower *SPF/LPF*

Recent reports described the enhancer activity exhibited by some plant introns when they are positioned near the transcription initiation site. In the same sense, the leader intron of the *Arabidopsis Cox5*c subunit showed this feature when fused to another related gene promoter, the one encoding the subunit 5b of the same protein complex. In order to establish whether this intron is a universal enhancer and if it is able to improve stress response, we have constructed a chimera where *SPF/LPF* were fused to this intron and cloned it directing the *gus* reporter gene activity. With these constructs, plants were transformed and analyzed by histochemical and fluorometric assays. It is important to note that histochemical assay is carried out in absolute darkness and that in this condition the *Hahb*-4 promoter has a maximal activity (data not shown). In Fig. 2, a comparison between the expression pattern in these plants and in plants from the genotype *SPF/LPF* is shown. The levels of expression measured by the non-quantitative method indicate that the gene is expressed in the same tissues/organs and developmental stages as in the plants bearing the construct without the intron, but more strongly. Regarding roots: *SPF/LPF* drive the expression in the central vascular cylinder and in the initiation of lateral roots (Dezar et al. 2005b, Fig. 2), whereas coloring in this organ of the novel plants is ubiquous and very strong, probably due to diffusion. Another difference in expression exhibited by these genotypes is the staining observed in flowers. Expression was almost undetectable in *SPF/LPF* genotype whereas in the *SPF:I:gus* activity could be well detected in sepals, stigma, anthers and pollen grains whereas petals remain uncolored.

Plants transformed with the constructs *SPF/LPF:I:Hahb4* do not show altered morphology

Aiming to obtain an improved phenotype using the potential of the *Hahb*-4 cDNA as stress tolerance donor, we have obtained transgenic plants bearing the constructs: *SPF/LPF:I:Hahb4*. When homozygous lines were isolated, a detailed phenotypic analysis was carried out. Germination time, root and stem length, number of rosette leaves, flowering time, siliquas number and seed production were evaluated in series of transgenic plants comparing with wild-type plants used as controls. In normal growth conditions, as defined in the Materials and methods, no significant differences were detected between genotypes (Fig. 3a–d). All the tested parameters remain constant and the little differences observed were lower than the standard deviations calculated from three independent experiments. These results led us to conclude that the novel constructs drive the expression of *Hahb*-4 at a level that is not enough to induce morphological changes in transformed plants resulting in an unchanged phenotype.

Table 1 Phenotypic characteristics of transgenic plants expressing *Hahb*-4 under the control of different promoters

Genotype and line name	No. of rosette leaves	Stem length (30 day-old)	No. of siliques (30-day-old)	Stem length (35 day-old)	No. of siliques (35-day-old)	Stem length (40 day-old)	No. of siliques (40-day-old)	Transpiration rate (μmol $m^{-2}s^{-1}$)	Gl (H_2O) (mmol $m^{-2}s^{-1}$)
35S:GUS	8	232 ± 18	20 ± 5	286 ± 20	48 ± 8	319 ± 22	57 ± 7	249 ± 23	17.8 ± 0.2
LPF:Hahb4-1	8	237 ± 16	16 ± 3	262 ± 15	48 ± 6	316 ± 17	56 ± 5	371 ± 15	30.7 ± 0.3
LPF:Hahb4-9	8	225 ± 12	18 ± 3	260 ± 17	47 ± 7	336 ± 22	57 ± 6	352 ± 33	28.4 ± 0.1
LPF:Hahb4-10	8	235 ± 18	14 ± 4	290 ± 14	46 ± 8	294 ± 19	58 ± 7	315 ± 25	23.8 ± 0.2
RD29:Hahb4-2	8	243 ± 20	17 ± 3	242 ± 14	49 ± 7	324 ± 20	58 ± 5	271 ± 11	21.4 ± 0.3
RD29:Hahb4-3	8	230 ± 14	18 ± 3	309 ± 16	49 ± 6	333 ± 19	56 ± 7	364 ± 9	20.0 ± 0.1
RD29:Hahb4-26	8	229 ± 21	16 ± 3	296 ± 19	48 ± 6	319 ± 17	57 ± 6	310 ± 38	23.9 ± 0.1
LEA:Hahb4-7	8	196 ± 17	15 ± 3	253 ± 18	47 ± 6	303 ± 18	57 ± 5	315 ± 34	26.1 ± 0.1
LEA:Hahb4-12	7	214 ± 14	17 ± 3	308 ± 15	49 ± 6	317 ± 19	57 ± 4	295 ± 10	22.3 ± 0.2
LEA:Hahb4-15	8	244 ± 18	17 ± 3	323 ± 20	49 ± 6	364 ± 22	56 ± 5	351 ± 4	29.2 ± 0.3
ATS1:Hahb4-1	8	226 ± 18	18 ± 3	311 ± 18	49 ± 6	324 ± 18	57 ± 5	290 ± 41	23.3 ± 0.3
ATS1:Hahb4-4	8	222 ± 18	16 ± 3	282 ± 14	48 ± 6	283 ± 17	56 ± 6	323 ± 22	24.5 ± 0.3
ATS1:Hahb4-18	8	215 ± 15	15 ± 3	289 ± 16	48 ± 6	302 ± 17	57 ± 5	347 ± 13	27.3 ± 0.2
35S:Hahb4-30[a]	8	160 ± 16	11 ± 3	269 ± 16	41 ± 7	279 ± 21	52 ± 7	370 ± 26	28.9 ± 0.2
35S:Hahb4-4[a]	8	178 ± 12	13 ± 3	240 ± 19	46 ± 6	278 ± 22	56 ± 7	302 ± 20	24.2 ± 0.1
35S:Hahb4-6[b]	11	12 ± 2	0	81 ± 7	0	149 ± 9	13 ± 2	394 ± 19	33.6 ± 0.1

Thirty two individuals from each genotype (named in the first column) were grown sharing tray with an equal number of *35S:GUS* individuals, used as controls, under standard conditions as described in Materials and methods. Phenotypic parameters were taken each 5 days (shown only those corresponding to 30–40 days). Number of rosette leaves was taken in the transition from vegetative to reproductive stage; Gl, stomatal conductance. The experiment was repeated at least three times with these lines and the data shown is the average of the replicates

[a] Low level expression transgenic lines

[b] High level expression *35S:Hahb4* plants present a delay in development and reach later their maximal height. These are representative examples of experiments using other transgenic lines for each construction showing similar results (not included)

Table 2 Survival rates of different genotypes of 4-week-old transgenic plants expressing *Hahb*-4

Genotype and line name	No. of T-DNA insertions	No. of plants per experiment	No. of survivors per experiment	% of survivors after rewatering	Transpiration rate (μmol $m^{-2}s^{-1}$)	Gl (H_2O) (mmol $m^{-2}s^{-1}$)
35S:GUS	1–4	24	2 ± 1	8	79 ± 9	4.7 ± 0.1
LPF:Hahb4-1	1	24	20 ± 2	83	134 ± 12	8.9 ± 0.3
LPF:Hahb4-10	2	24	19 ± 2	79	156 ± 16	10.1 ± 0.2
SPF:I:Hahb4-1	1	24	24 ± 1	100	98 ± 6	5.9 ± 0.2
SPF:I:Hahb4-2	2	24	24 ± 1	100	91 ± 10	6.2 ± 0.1
RD29:Hahb4-2	3	24	19 ± 2	79	106 ± 17	7.3 ± 0.3
RD29:Hahb4-3	5	24	18 ± 1	75	109 ± 3	7.0 ± 0.2
RD29:Hahb4-26	3	24	19 ± 2	79	94 ± 11	7.1 ± 0.1
LEA:Hahb4-7	2	24	16 ± 1	67	115 ± 12	7.2 ± 0.3
LEA:Hahb4-12	1	24	15 ± 1	63	107 ± 6	7.4 ± 0.3
LEA:Hahb4-15	1	24	18 ± 2	75	98 ± 8	5.9 ± 0.2
ATS1:Hahb4-1	2	24	15 ± 1	63	95 ± 7	6.3 ± 0.1
ATS1:Hahb4-4	1	24	12 ± 1	50	103 ± 16	7.5 ± 0.3
ATS1:Hahb4-18	5	24	19 ± 2	79	120 ± 4	8.4 ± 0.3
35S:Hahb4-30[a]	2	24	24	100	103 ± 11	6.0 ± 0.1
35S:Hahb4-6[b]	1	24	24	100	124 ± 15	8.1 ± 0.3

Average numbers (three independent experiments) of 4-week-old *Hahb*-4 expressing transgenic plants surviving after exposure to water stress. Each set of transgenic plants shared the tray with control ones

[a] Low level expression transgenic lines

[b] High level expression *35S:Hahb4* plants (line 6); Gl, stomatal conductance. These are representative examples of experiments using various transgenic lines for each construct. The same approach was taken using WT plants as controls, yielding similar results

Water stress tolerance conferred by *SPF/LPF:I:Hahb-4* is as high as the conferred by *35S:Hahb4*

We were interested in evaluating the capacity for drought resistance of the novel genotypes of transgenic plants during vegetative and reproductive stages of development. For this purpose, transgenic and wild-type *Arabidopsis* plants were grown in pots intercalated in the same tray. After cold treatment for dormancy break, the pots were watered by adding 1 l of water to the trays and then transferred to a culture chamber. The first symptom of water deficit was a diminution in growth rate. Upon prolonged water deficit, damage became evident in wild-type plants. Twenty to 25 days later, leaves of wild-type plants became wilted and curled, whereas transgenic plants were affected a few days later. The drought treatment was extended for five days until severe damage was visible. At this stage, plants were watered and 2 days later survivants were observed and counted in the different populations. Compared with the survival rate of non-transformed plants, all the transgenic genotypes were clearly more tolerant. Fig. 3e and Table 2 illustrate experiments carried out with independent lines subjected to water deficit as described in the Materials and methods. In all cases, the percentage of plants surviving under severe stress conditions is considerably higher for transgenics compared to the non-transformed genotypes. However, plants transformed with *SPF:I:Hahb4* show a survival rate under severe conditions as high as that observed in plants transformed with the constitutively expressed construct. Membrane stability of the different genotypes was measured in order to establish if this parameter could explain the observed stress tolerance; however, no significant differences that indicate that tolerance is not achieved by this mechanism were observed (Table 3). In the same way transpiration rates and stomatal conductance of these plants do not exhibit significant differences with those measured in WT plants (Table 3, last columns) and cannot explain the observed tolerance. These results led us to ask for the mechanism responsible for such a performance in these transgenic plants. Do they express higher levels of *Hahb*-4 or they express this gene faster after induction by stress? Is the increase transcriptional, translational or a combination of both?

SPF/LPF:I induces higher levels of transcript expression compared to *SPF/LPF* in control conditions and increases at the same rate after a stress treatment

With the aim of testing the different hypothesis, kinetics of expression was analyzed in transgenic genotypes. Plants from several independent lines belonging to the genotypes *35S:Hahb4*, *SPF:Hahb4*, *LPF:Hahb4*, *SPF:I:Hahb4* and

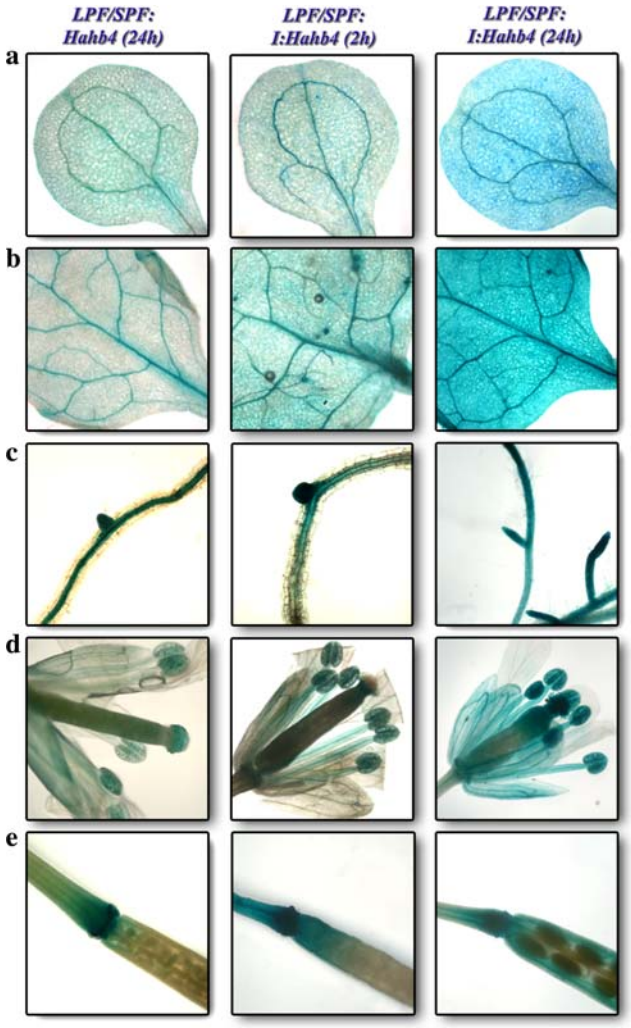

Fig. 2 Comparison between expression patterns of transgenic plants bearing *SPF/LPF:I:gus or SPF/LPF:gus* constructs. Histochemical detection of GUS activity after 24 h of enzymatic activity in *Arabidopsis* transgenic plants transformed with *SPF/LPF:gus* (*left column*) or with *SPF/LPF:I:gus* during the same time (*right column*), or during 2 h (*middle column*). **a** Cotyledons, **b** 4-week-old leaves, **c** Three-week-old roots, **d** Inflorescences, **e** Siliques

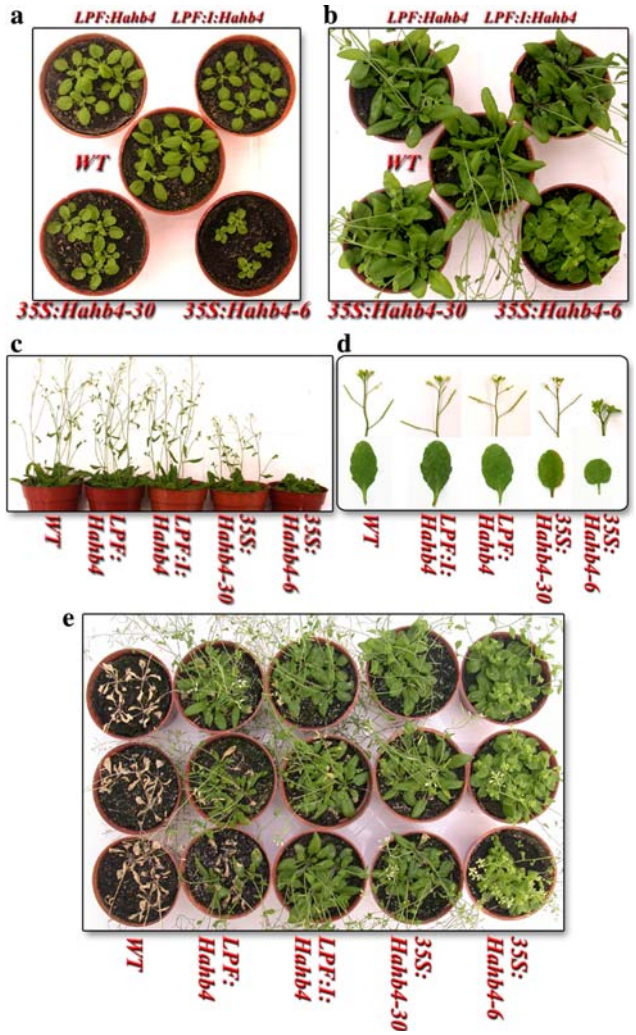

Fig. 3 Phenotype of *LPF:I:Hahb4* transgenic plants. **a**, **b** Aerial view of plants (20-day-old and 30-day-old, respectively) grown in control conditions. **c** A lateral view of 45-day-old plants. **d** A detail of leaves and inflorescences of the same plants. **e** Aerial view of plants subjected to a severe water stress and then irrigated. WT, non transformed plants; *LPF:Hahb4*, *LPF:I:Hahb4* and *35S:Hahb4*: plants transformed with the corresponding constructs as described in Materials and methods. The photograph was taken 2 days after re-watering. High-expressing *35S:Hahb4* is represented by line 6 (*35S:Hahb4*-6) and low-expressing *35S:Hahb4* by line 30 (*35S:Hahb4*-30)

LPF:I:Hahb4 were subjected to drought stress or grown in normal conditions. Samples were harvested at several times to measure transcript levels (Fig. 4). As it can be observed in the figure, transcript levels in non-stressed 3-week-old plants (time 0) are 17 times higher with the intron-bearing construct than in those lacking it, whereas comparing the constitutive construct with *SPF:I:Hahb4* we found a ratio of 3 times for high expressing plants and 1.6 times for plants with "low expression". Except for the constitutive genotypes that maintain almost constant transcript levels along the experiment (additional 20 days) as expected, the two inducible genotypes show a very similar increasing rate, leading to higher concentrations of *Hahb*-4 in *SPF:I:Hahb4* plants, high enough to generate tolerance and low enough to avoid being accompanied by morphological defects.

Aiming to determine if in addition of being a transcriptional enhancer the intron acts as a translational one, and since we were unable to measure protein (Hahb-4) concentration, we performed an experiment comparing GUS activity in transgenic plants treated with ABA. Figure 5 shows the kinetics of appearance of GUS activity measured by fluorometry in 2-week-old plants treated with 100 µM ABA. In normal growth conditions, transcript levels for *gus* were (in relative units): 1.00 ± 0, 26 for *SPF/LPF:gus*, $15.73 \pm : 0.97$ for *SPF/LPF:I:gus*, $0{,}06 \pm 0.02$ for *pBI101.3*

Table 3 Phenotypic characteristics of transgenic plants expressing *Hahb*-4 under the control of *SPF or SPF:I* promoters

Genotype and line name	No. of rosette leaves	Stem length (30-day-old)	No. of siliques (30-day-old)	Stem length (35-day-old)	No. of siliques (35-day-old)	Stem length (40-day-old)	No. of siliques (40-day-old)	Transpiration rate (μmol $m^{-2} s^{-1}$)	Gl (H_2O) (mmol $m^{-2}s^{-1}$)
35S:GUS	8	226 ± 17	19 ± 5	276 ± 20	47 ± 7	312 ± 20	59 ± 9	249 ± 23	17.8 ± 0.2
SPF:Hahb4-1	8	236 ± 16	18 ± 4	269 ± 14	48 ± 6	316 ± 15	56 ± 6	354 ± 22	30.1 ± 0.3
SPF:Hahb4-4	8	231 ± 12	17 ± 3	279 ± 16	48 ± 7	306 ± 20	57 ± 8	326 ± 12	25.1 ± 0.3
SPF:Hahb4-7	8	229 ± 18	16 ± 4	276 ± 15	47 ± 8	299 ± 19	58 ± 8	308 ± 33	25.8 ± 0.1
SPF:I:Hahb4-2	8	230 ± 18	17 ± 3	277 ± 18	46 ± 6	312 ± 22	56 ± 7	307 ± 29	25.2 ± 0.2
SPF:I:Hahb4-4	8	226 ± 16	18 ± 4	274 ± 17	45 ± 7	315 ± 16	57 ± 6	289 ± 13	21.7 ± 0.1
SPF:I:Hahb4-9	8	229 ± 15	16 ± 3	270 ± 14	48 ± 5	308 ± 17	55 ± 7	292 ± 10	23.9 ± 0.3
35S:Hahb4-8[a]	8	167 ± 16	11 ± 3	252 ± 14	41 ± 6	276 ± 18	52 ± 6	365 ± 08	29.6 ± 0.3
35S:Hahb4-11[a]	8	179 ± 12	12 ± 4	254 ± 16	40 ± 5	278 ± 19	54 ± 7	368 ± 30	29.3 ± 0.2
35S:Hahb4-6[b]	11	12 ± 2	0	74 ± 7	0	139 ± 9	13 ± 2	394 ± 11	34.5 ± 0.2

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 Materials and methods. Phenotypic parameters were taken every 5 days (shown only those corresponding to 30–40 days). The number of rosette leaves was determined in the transition from vegetative to reproductive stage; Gl, stomatal conductance. The experiment was repeated at least three times with these lines and the data shown is the average of the replicate

[a] Low level expression transgenic lines

[b] High level expression *35S:Hahb4* plants present a delay in development and reach later their maximal height. These are representative examples using other transgenic lines for each construction showing similar results (not included)

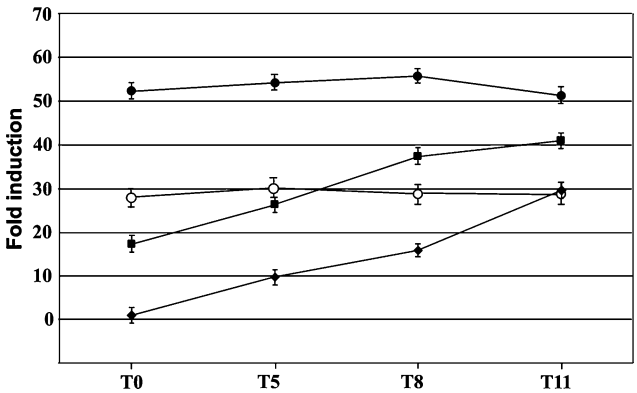

Fig. 4 Kinetics of expression driven by *SPF/LPF:I:Hahb4*. Transcript levels of *Hahb*-4 expressed by transgenic plants subjected to water stress and measured by real time qRT-PCR. Transcript level for the construct *LPF/SPF:Hahb4* in control conditions was taken arbitrary as a one, and the others graphed as a ratio referred to this value, taken as standard. Samples were collected at 0 (control growth conditions), 5, 8 and 11 days after applying water stress. The analyzed genotypes were high-expressing *35S:Hahb4* (*filled circle*), low-expressing *35S:Hahb4* (*open circle*), *LPF/SPF:Hahb4* (*filled diamond*) and *LPF/SPF:I:Hahb4* (*filled square*). Standard deviations were calculated from three independent experiments done with biological triplicates each one

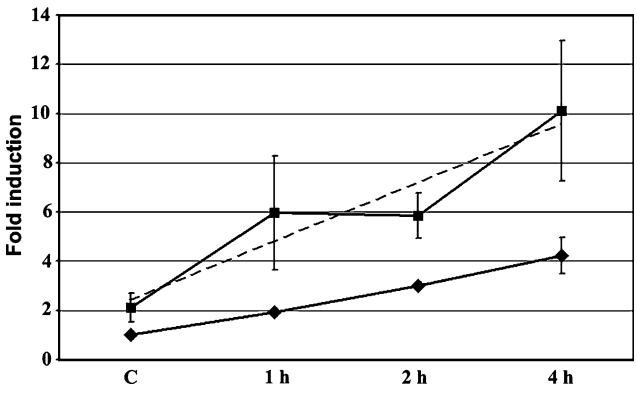

Fig. 5 Kinetics of expression driven by *SPF/LPF:I* and *SPF/LPF* in transgenic plants treated with ABA. Fluorometric measurements of protein extract samples prepared from transgenic plants (3-week-old) treated with ABA. Fluorometric activity in plants bearing the construct *LPF/SPF:GUS* in control conditions was taken arbitrary as a one, and the others graphed as a ratio referred to this value, taken as standard. Samples were collected at *C* (control growth conditions), 1, 2 and 4 h after applying the hormone. The analyzed genotypes were *LPF// SPF:Hahb4* (*filled diamond*) and *LPF/SPF:I:Hahb4* (*filled square*). Standard deviations were calculated from three independent experiments performed with three biological replicates each one

(used as negative control) and 54, 44 ± 2.35 for *35S:gus*. After ABA treatment, GUS activity increased linearly in both genotypes indicating that transcription and translation exhibit the same behavior in what concerns inducibility. Regarding the kinetics it seems that plants bearing *SPF/LPF:I:Hahb4* augmented transcript levels faster than plants bearing *SPF/LPF:Hahb4*. However, *P* value = 0.06 in test *T* indicated that the observed difference is not significant.

Discussion

In a previous work we described obtaining of transgenic *Arabidopsis* plants able to tolerate strong water stress by overexpressing the sunflower HD-Zip gene, *Hahb*-4. Unfortunately, these plants showed also a delay in development and some undesired morphological characteristics.

These results were similar to those obtained by other authors when overexpression of a gene encoding a transcription factor or another type of protein involved in stress response was used as a strategy to obtain stress tolerant transgenic plants (Gilmour et al. 1998; Kasuga et al. 1999, 2004; Hsieh et al. 2002; Hjellström et al. 2003; Sunkar et al. 2003).

On the other hand, when plants were transformed with the same genes controlled by inducible promoters instead of being overexpressed, undesired phenotypic characteristics disappeared, but stress tolerance diminished. Besides, plants expressing the transgene constitutively but at lower levels showed a good stress tolerance associated with a very slightly changed phenotype. However, low level expressing plants were difficult to obtain; among approximately 50 lines analyzed in two independent transformation experiments only two in one of them and three in the other one showed a diminished expression level (not shown).

Based on the results obtained from Pellegrineschi et al. (2004) and ourselves, we planned to generate tolerant transgenic plants by expressing *Hahb*-4 in an inducible way so as to improve its expression in the desired time. We selected a set of promoters to test the behavior of transgenic plants when the expression of *Hahb*-4 was controlled by them. *GmPM9* encoding a LEA protein, known to be inducible by dehydration, was selected first (Lee et al. 2000). Although the promoter of this soybean gene is not deeply characterized, it presented the advantage of belonging to an agronomic interesting crop, potentially useful in near future. *Ats1A* was chosen as a light inducible promoter (Dedonder et al. 1993). *Rd29* is a very well characterized promoter inducible by drought and salinity stresses (Yamaguchi-Shinozaki and Shinozaki 1993; Narusaka et al. 2003). It was used to drive expression of DREB1A, instead of the *35S*, in order to obtain fertile stress tolerant transgenic plants (Kasuga et al. 1999, 2004).

Our observations indicate that all these promoters driving *Hahb*-4 expression have a good performance, i.e. plants are healthy, show no developmental delay and their seed production closely resembles that one of non-transformed plants. In spite of this fact, the achieved stress tolerance was not as high as that one obtained with the constitutive construct; indicating that the rate of induction, or the total amount of the transgenic protein translated are not enough to protect the plant against strong stresses. Together, the results suggest that a faster or higher induction must be achieved to obtain stress-tolerant plants without undesired characteristics.

Therefore, we looked for an enhancer sequence able to improve the performance of any promoter. This enhancer must be unable to drive expression by itself. We found the leader intron of *Arabidopsis Cox5*c as a good candidate because it was competent to enhance the promoter of *Cox5*b activity conserving the tissue/organ specific pattern of expression driven by it (Curi et al. 2005). However, both of the nuclear encoded mitochondrial genes lack the typical eukaryotic TATA box, and thus we had some doubts on the chosen strategy because *Hahb*-4 possess a typical eukaryotic promoter with a TATA box (Gago et al. 2002). We inserted the intron in the correct orientation and position in the promoter of *Hahb*-4 and transformed plants with this construct. As a control of possible changes in the expression pattern of *Hahb*-4 we obtained plants with this chimerical promoter driving the *gus* reporter gene expression. These plants showed an enhancement of inducible expression in roots and flowers and at a lesser extent in leaves, cotyledons and meristematic regions. No changes were observed in stems and pedicels, tissues in which a total lack of expression had always been observed. Positioning the intron in the opposite orientation fully abolished *SPF/LPF* driven expression (data not shown). Together, these results suggested that the intron is able to enhance the *Hahb*-4 promoter activity without changing the tissue specific expression.

Transgenic plants (*SPF:I:Hahb4*) were indistinguishable from wild type in normal growth conditions and showed a stress tolerance as high as the one shown by the constitutive genotype. Our experimental data indicated that the stress tolerance achieved was due to a combination of effects both on transcription (Fig. 4) and translation (Fig. 5). A higher level of expression of the transgene is observed in the fusion constructs compared with that of the constructs lacking the intron. However, these higher levels seem to be not high enough to produce morphological alterations. Moreover, transcript and protein levels increased at the same rate in *SPF:Hahb4/gus* and *SPF:I:Hahb4/gus* after induction in the intron of plants and lead to constant major levels of the transcription factor. On the other hand, the low constitutive expressing plants do not show an altered phenotype and, as expected, maintain the same transcript levels with or without the drought treatment. Transcript levels in these plants were higher than in *SPF:I:Hahb4* plants under control conditions but the last ones caught up with and even exceeded this level during the stress treatment. The experimental data indicated that is necessary to reach a particular level of expression in stress conditions and not to exceed it in control ones to obtain a tolerant normal phenotype. This level is not achieved by the inducible genotypes on time. Both, inducible and low level expression constitutive genotypes exhibit transpiration rates and stomatal conductances similar to those measured in WT plants indicating that tolerance is not achieved by this mechanism (lower transpiration). Accordingly, Alvin et al. (2001) reported that transgenic plants expressing BiP presented enhanced drought tolerance even with higher rates of transpiration. These authors suggested that under progressive drought conditions the photosynthesis rate was much less affected than in control plants leading to a reduced utilization of photoassimilates

that could account, at least in part, for the increase in solute concentration in transgenic plants. It is likely that a similar pathway is followed by our transgenic genotypes, considering that genes encoding key enzymes in osmoprotectants biosynthesis are up-regulated in transgenic plants (Manavella et al. 2006) while CO_2 assimilation is maintained (data not shown).

The action mechanisms in which plant introns are involved to enhance transcriptional and/or traslational activity of promoters is still unknown. Several authors suggested that certain introns may contain transcriptionally active regulatory elements (Gidekel et al. 1996), whereas others act post-transcriptionally (Rose and Last 1997). It has recently been proposed that many introns would act by increasing the processivity of the transcription machinery (Rose 2004). Several mechanisms of action coexist, but none of them is fully understood. Regarding our results (Figs. 4, 5), it is clear that a transcript accumulation occurs, since the translation is not as enhanced as transcription when the intron is inserted in the construct (17-fold in *Hahb*-4 transcript level and 16-fold for *gus* in control conditions while only twice in GUS activity, indicative of translation). On the other hand, it seems that *LPF/SPF* promoter conserves its capability and rate of induction in the presence of ABA or when plants are stressed, independently from the intron action. Together, these results indicate that the better performance against drought achieved by the chimerical construction is due to the basal content of *Hahb*-4 transcript, more than to an increased capability of induction.

From a biotechnological point of view, we can conclude that the strategy of constructing chimeras with such introns is useful to enhance an inducible promoter activity without changing morphological or productive characteristics of the plants. From a biological viewpoint, our results support the existence of a high conservation of these still unknown mechanisms between species. The chimera was constructed with a segment of a sunflower promoter and an *Arabidopsis* intron and used to transform *Arabidopsis* plants where it was recognized and processed both driving *gus* or the sunflower *Hahb*-4 expression. It was also recognized in transiently transformed sunflower (data not shown).

Further studies will be necessary to elucidate in which way this expression occurs so efficiently. In spite of the poor understanding about the action mechanism of the chimera, a powerful biotechnological tool in order to transform plants was obtained, analyzed and is actually ready to be used.

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