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Seed-specific expression patterns and regulation by ABI3 of an unusual late embryogenesis-abundant gene in sunflower

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Abstract

We cloned the genomic sequences that correspond to a previously described group 1 late embryogenesis-abundant (*Lea*) cDNA from sunflower: *Ha ds10*. The *Ha ds10 G1* gene had structural and gene-expression features that depart from those of other group 1 *Lea* genes. An intron was present at a conserved position but showed a much larger size (1024 bp). Transcription from the *Ha ds10 G1* promoter was strictly seed-specific and it originated from at least two close initiation sites. The mRNAs accumulated from stages of embryogenesis that preceded seed desiccation. *Ha ds10 G1* mRNA accumulation was moderately induced, by exogenous abscisic acid treatments, in immature seeds but not induced in seedlings. We observed unprecedented changes in *Lea* mRNA localization associated with seed desiccation: the homogeneous tissue distribution of *Ha ds10 G1* mRNAs, which was characteristic of immature embryos, evolved later in embryogenesis to an asymmetric distribution within the cotyledons, with preferential mRNA accumulation in the cells of the palisade parenchyma and provascular bundles. We also showed that, in sunflower embryos, the *Ha ds10 G1* promoter could be transiently activated by the *Arabidopsis* ABI3 transcription factor. We discuss the significance of these results regarding hypotheses of regulation and function of plant genes from the same family.

Introduction

The zygotic embryo desiccates towards the end of plant embryogenesis. Several gene families are coordinately induced concomitant with embryo desiccation, with transcripts depicting maximal accumulation during the post-abscission stage (e.g. [19], reviews [9, 11], and references therein). These genes are termed *Lea*: late embryogenesis-abundant. A subset of *Lea* genes is also expressed, subsequent to seed germination, in response to water stress in vegetative tissues. Some *Lea* genes are also induced by application of exogenous abscisic acid (ABA), a hormone involved in signal transduction in the water and other stress responses of plants (reviewed in [9, 16, 32]). The proteins encoded by *Lea* genes have been classified into at least six different sequence homology groups. These groups are conserved between monocot and dicot plants, and very likely represent functionally distinct, and differentially regulated, subsets of genes/proteins [12]. Structural analyses of the *Lea* proteins, as well as some studies of their localization and abundance, have set forward functional hypotheses that associate these proteins with protection and recovery from the molecular damage, induced by water removal and/or ion concentration, as a result from embryo desiccation ([28], revision in [12]). Recent functional studies in yeast [20] and rice transgenic plants [35] support these hypotheses. Other research has focused in the regulation of *Lea* genes during embryogenesis and the role of ABA. These studies have identified different involved *cis* elements and *trans*-acting factors (e.g. [17, 23, 18]). Genetic analyses have indicated, in some

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AJ224116.

cases, that the same *trans*-acting factor, such as ABI3 in *Arabidopsis*, is involved in the regulation during embryogenesis of different members of the *Lea* gene families [26]. The ABI3 transcription factor has been cloned and shown to participate in regulation of different gene programs that are specific to plant zygotic embryogenesis [15, 26]. The *cis* elements that mediate this regulation have not always been determined [26]. Recent results of immunomodulation of ABA in seeds confirmed a key role for this hormone in the regulation of gene expression associated with embryo desiccation and desiccation tolerance [27]. However, the coordinate regulation of the *Lea* genes is most likely the result of distinct, temporarily overlapping, control pathways. Systematic and detailed analyses of the regulation and expression patterns of *Lea* genes are still scarce for most gene families and plant species.

We have reported the isolation from sunflower dry seeds of *Ha ds10*, a cDNA encoding a polypeptide belonging to the group 1 of *Lea* proteins [1, 12]. This group contains proteins characterized by a very high proportion of charged residues and Gly, which results in high hydrophilicities and predicts mostly amorphous (random coil) structures, with several short helical regions interspersed. This has led to suggestions of their functional involvement in water binding during embryo desiccation [12]. The most notable sequence feature of group 1 *Lea* proteins is that the homology among members from diverged plant genera extends through most of the length of the molecules. Much of the sequence variation is caused by repeated motifs in the center of the molecule, which results in long and short forms of these proteins that are present within the same species [12, 31]. The predicted protein for the *Ha ds10* cDNA shared these sequence features, but it was also found to be the most diverged member of the group 1 *Lea* gene family in plants [31]. This raised our interest in investigating its regulation and expression patterns during zygotic embryogenesis. In this work, we isolated the genomic sequences corresponding to the original *Ha ds10* cDNA. We observed additional features that are consistent with the notion that this gene is an unusual and highly diverged member of the group 1 *Lea* family: its coding sequence was interrupted by a very long intron that is located at a conserved position. In sunflower, transcription from the *Ha ds10 G1* promoter was seed-specific, and we show that it might be controlled by ABI3-like factors. Similar to some *Lea* genes belonging to the group 1 (i.e., the *Arabidopsis* Em1 [14, 26]), *Ha ds10 G1* was activated before desiccation, but the *Ha ds10 G1* promoter did not substantially respond to exogenous ABA. Finally, mRNA localization experiments in sunflower embryos suggest that, during desiccation, the ds10 protein could be prevalent in tissues that are associated with the deposition and/or mobilization of the reserve proteins: the vascular bundle and palisade parenchyma cells in the cotyledons. All these results provide additional evidence for the differential regulation, evolutionary divergence, and possible functional specialization of plant genes belonging to the group 1 *Lea*.

Material and methods

Gene isolation and sequencing

The genomic library [6], as well as conditions for DNA hybrization, probe washings, phage isolation and subsequent subcloning have been described before [1]. The *Ha ds10 G1* sequences were obtained on both strands of DNA according standard methods ([1] and references therein). Nucleotide sequences were analyzed with the GCG (University of Wisconsin) software package.

RNA assays: primer extension and RNAse A protection

Total RNA was prepared from staged sunflower embryos and seedlings from plant material (*Helianthus annuus* L. cv. Sunweed) grown under controlled environment. Conditions for plant growth, exogenous abscisic acid treatments (for 24 h, with 10 μM ABA) and RNA preparation have been previously described ([1] and references therein). The oligonucleotide primer 5′-CTCCTGTTCCGGAATTTTGCGTGT-3′ (non-coding strand of *Ha ds10 G1* between +25 and +48) was labelled with ^{32}P and hybridized to 25 μg of total RNA from staged sunflower embryos, using procedures essentially as described by Domon *et al.* [10] Hybridization with this primer was at 62 °C in 14 mM Tris-HCl pH 8.0, 240 mM KCl, 0.9 mM $MgCl_2$ and 0.45 mM DTT. Hybrids were extended with AMV reverse transcriptase (Pharmacia Biotech) for 90 min at 42 °C. Extension products were analyzed in 6% PAGE sequencing gels, alongside sequence reactions of *Ha ds10 G1* DNA produced from the same primer. The RNA samples were also analyzed by RNAse A protection, after hybridization with a RNA probe

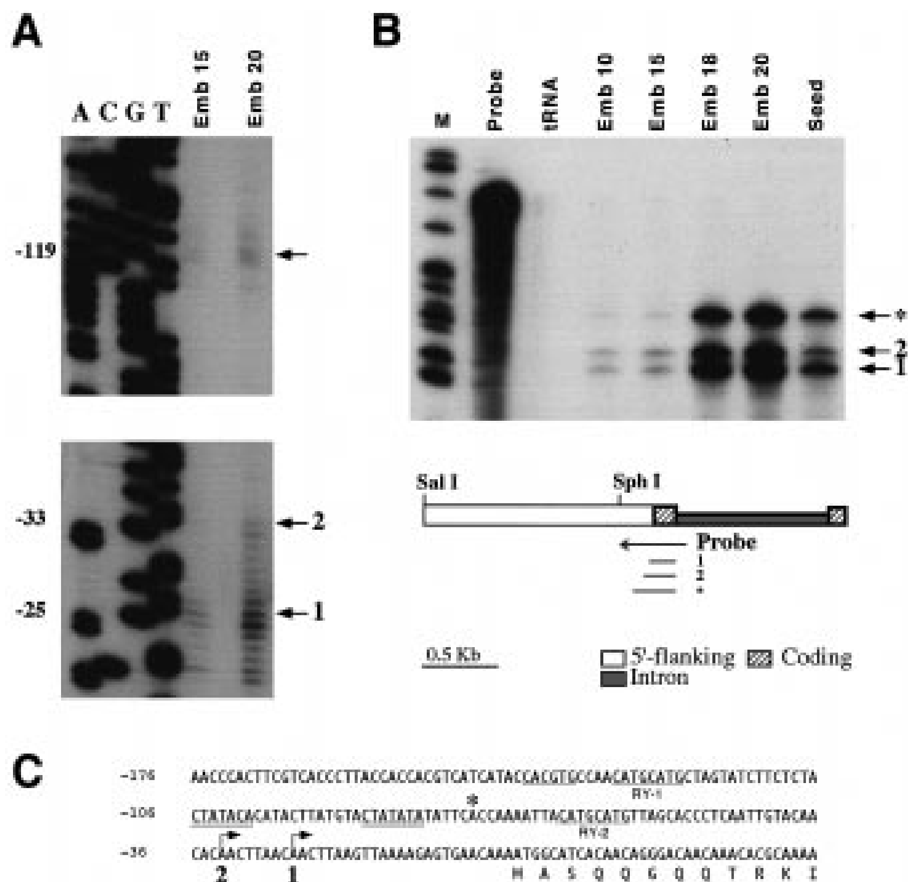

Figure 1. Transcriptional initiation and mRNA accumulation patterns of *Ha ds10 G1* during zygotic embryogenesis. Different total RNA samples were analyzed by primer extension (A) and RNAse A protection (B). The RNA samples were from embryos (Emb) at different developmental stages (numbers indicate dpa), and yeast tRNA, used as negative control in the protection assays. The extended DNA (A), or protected mRNA molecules (B) were separated by electrophoresis in polyacrylamide gels (see Materials and methods for details). Arrows with numbers mark bands that indicate the transcription initiation sites confirmed by both assays (1 and 2). Non-coincident bands are shown by arrows without numbers (for details see text). The scheme in the bottom of panel B depicts a restriction map of the 5′-flanking region of *Ha ds10 G1*, the probe used for the RNAse A protection assays, and a graphic interpretation for the protected RNA bands. DNA size markers were: the sequencing reactions (A, C, G, T) in panel A, and *Hpa*II digested pBR322 (M, panel B). Numbers in panel A mark positions on the *Ha ds10 G1* sequence. Panel C depicts the position of the transcription start sites (1, 2 and *), as well as other salient features of the *Ha ds10 G1* genomic sequence. Putative TATA-boxes are double-underlined. Other possible *cis*-acting elements are underlined: the RY repeats (1 and 2), and one of CACGTG consensus sequences further described in the text. The 5′ end of the coding region is indicated by translation below the nucleotide sequence.

(riboprobe). This riboprobe corresponds to the non-coding DNA strand of *Ha ds10 G1*, and it extends from +212 (in the intron) to the *Sph*I site at −121. This riboprobe was prepared by *in vitro* transcription, with T3 RNA polymerase, from a DNA plasmid template (ds10G1S3Δ4.4) that contained the *Ha ds10 G1* sequences between *Sal*I (−1576), and +212 (adjacent to *Bam*HI in the polylinker), cloned in the vector pBluescript SK+ (Stratagene). Conditions for riboprobe preparation, hybridization, digestion with RNAse A, and subsequent gel analyses of protected RNA fragments were previously described ([6] and references therein).

Transient expression in sunflower embryos

The assays were carried by transient expression in embryos 17–20 days after anthesis (dpa), bombarded with DNA-coated gold particles using a ‘Biolistic PDS-1000 He’ device (BioRad). Prior to bombardment the embryos, peeled from sterilized seeds, were sectioned in two (between cotyledons) and cultivated in the dark at 25 °C, for 2–4 h (curved surface up) on Petri dishes

containing MS medium with 2% sucrose, 0.5 M sorbitol and 8% agar. Six half-embryos were used per shot. Bombardment conditions were: rupture disks, 10.7 MPa; distance from rupture disk to macro-carrier, 8 mm; distance from macro-carrier to stopping screen, 6 mm; and distance to target tissue, 6 cm. As microprojectiles for DNA delivery we used gold particles (BioRad, 1.6 μm) coated with a mixture of three plasmids (reporter, effector and reference plasmids, see below). Plasmid DNA was prepared using the Quantum midi prep kit (BioRad), and precipitated onto the gold particles by the procedure of Chern *et al.* [5]. The mixture included, per shot, reference (0.2 μg), effector (1 μg) and reporter (1 μg) plasmids. In samples without effector, we substituted it for the same amount of plasmid pJIT82 [3]. The reference plasmid, used to normalize reporter GUS activity with luciferase, was pDO432 [25]. Bombarded embryos were cultivated in the dark for 24 h at 28 °C. GUS activity, from the reporter plasmids, was assayed and normalized to luciferase activity essentially as follows: bombarded embryos were frozen in liquid nitrogen, ground to fine powder and homogenized in luciferase lysis buffer (Luciferase Reporter Gene Assay Kit, Boehringer). Luciferase assays were performed with 1 μl of extract, diluted with 29 μl luciferase lysis buffer, and mixed with 65 μl luciferase substrate (same kit). Light emission was measured for 10 s in a Turner TD-20/20 luminometer. GUS assays were performed for 1 h at 37 °C, with 3 μl of luciferase extract in 200 μl of GUS assay buffer [21] with 1 mM MUG and 20% methanol [22]. Fluorescence was measured as described [21, 6]. The normalized GUS activity is the reading of the GUS assay multiplied by 10 and divided by the reading of the luciferase assay.

Effector and reporter plasmids

The effector plasmid, p35S::ABI3, was constructed by substituting the PvALF1 cDNA, in plasmid pALF (a derivative of pJIT82 [3]), for the ABI3 cDNA, obtained from plasmid pcabi3-4F [15]. The PvALF cDNA was excised from pALF with *Hin*dIII and *Eco*RI. The ABI3 cDNA was inserted in its place as a *Xba*I-*Eco*RI fragment. Ligation was performed after making blunt the non-compatible DNA ends with standard Klenow treatments [29]. We used two different reporter plasmids: pSKds10F2 (F2) and pSKds10F2ΔRY1 (F2Δ). Plasmid pSKds10F2 is a ds10::GUS::ds10 chimeric gene cloned in the vector pBluescript SK+ (Stratagene). This plasmid contains the *Ha ds10 G1* sequences, between the *Sal*I site at −1576 to +98, fused in frame to the bacterial β-glucuronidase (GUS) coding region in vector pBI101.2 [21]. In addition, it contains the *Ha ds10 G1* sequences between the *Pvu*II site in the second exon (+1205) and a 3′-distal *Eco*RI site located at ca. +4670. These sequences replaced the nopaline synthase (*nos*) terminator sequences in pBI101.2. The final expression cassette was transferred from the binary vector to pBluescript SK+ as a *Sal*I–*Eco*RI DNA fragment (see scheme in Figure 4B). Plasmid pSKds10F2ΔRY1 was derived from pSKds10F2, by deletion of 5 nucleotides between −126 and −122 in the *Ha ds10 G1* promoter.

In situ *RNA localization*

Sunflower embryos were fixed, included in paraffin, sectioned, and hybridized with riboprobes essentially as described by Molinier [24], with the modifications that follow. We optimized the fixation procedure for sunflower embryos of different ages (among 8 and 28 dpa). The final protocol included longer fixation times (from 18 h to 4–5 days, increasing with age), at 4 °C in: 0.1 M potassium phosphate (pH 7.2), 4% paraformaldehyde, 0.5% glutaraldehyde, and 3% sucrose. Dehydration steps in ethanol (consecutively: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 85%, 95%, and twice 100%) were also prolonged to 30–90 min each. Paraffin inclusion of fixed embryos was performed by treatment with 100% toluene (1–3 h, two times); followed by toluene/paraffin (1:1) at 65 °C for 6–15 h; and ended by five consecutive inclusions in paraffin, at 60 °C for 5–15 h. After the last inclusion step, paraffin blocks were cooled uniformly as fast as possible. Embryo sections were prepared, and hybridized to digoxigenin-labelled ds10-3′ and 18S rRNA riboprobes. Prehybridizations and hybridizations were performed at 45 °C. After hybridization, we included a washing step (for 30 min at 37 °C) in NTE (500 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA) with 20 μg/ml RNAse A, to increase the gene specificity of the hybridization with the ds10-3′ (−) riboprobe. This step is preceded and followed by washes at 37 °C, in NTE, without RNAse A (3 × 10 min and 4 × 15 min, respectively). We detected RNA hybrids with alkaline phosphatase. Tissue sections (8–10 μm thick) were photographed under Nikon (SMZ-U) or Olympus (BX40) microscopes.

To prepare the riboprobes used in the mRNA localization experiments, the *Ha ds10 G1* sequences

between +870 and +1592 were cloned, as a *Hin*dIII fragment of 723 bp, in pBluescript SK+. This resulted in plasmid ds10G1S1, which was used as a template for *in vitro* transcription. Transcripts were labelled with DIG-UTP using the DIG RNA labelling kit (Boehringer) and conditions suggested by the manufacturer. The probe ds10-3′ (−) was prepared by transcription using T3 RNA polymerase, after digestion of ds10G1S1 with *Pvu*II. This probe corresponds to the antisense strand of *Ha ds10 G1* between positions +1202 (the *Pvu*II site in the second exon) and +1592 (in the 3′-flanking region). A second *Ha ds10 G1* probe, ds10-3′ (+), was prepared after linearization of ds10G1S1 DNA with *Bam*HI (in the vector polylinker), and transcription with T7 RNA polymerase. The probe ds10-3' (+) contains the *Ha ds10 G1* sequences between +870 and +1592 (sense strand). The 18S RNA riboprobe was prepared by *in vitro* transcription of the 'G' *Eco*RI fragment from the radish gene [8], after subcloning it in pBluescript SK+.

Results

Isolation, characterization and seed expression of the ds10 *genomic sequences*

The *Ha ds10 G1* gene was isolated by hybridization screening of a λGEM-11 library from sunflower [6]. Using as a probe the complete *Ha ds10* cDNA [1], several highly homologous genomic clones were obtained. One of these clones, *Ha ds10 G1*, was shown by nucleotide sequencing to correspond to the original cDNA used for library screening. The determined nucleotide sequence included 1576 bp from the promoter and 5′-flanking sequences, and 553 bp of genomic 3′-flanking sequences (not present in the cDNA). The genomic (*Ha ds10 G1*) sequences that corresponded to the cDNA (493 bp [1]) were identical, with the exception of a dinucleotide inversion at position +1176/1177. We have revised our former data and confirmed that this inversion was also present in the original cDNA. The updated (cDNA) and new sequence data have been submitted to EMBL database (accession numbers X59699 and AJ224116). Comparison between the ds10 cDNA and the *Ha ds10 G1* sequences also identified a single intron, from +146 to +1169, defined by the boundary consensus sequence that flanks introns of plant genes [30]. The intron is placed in a position within the coding sequence that is conserved among other members of the

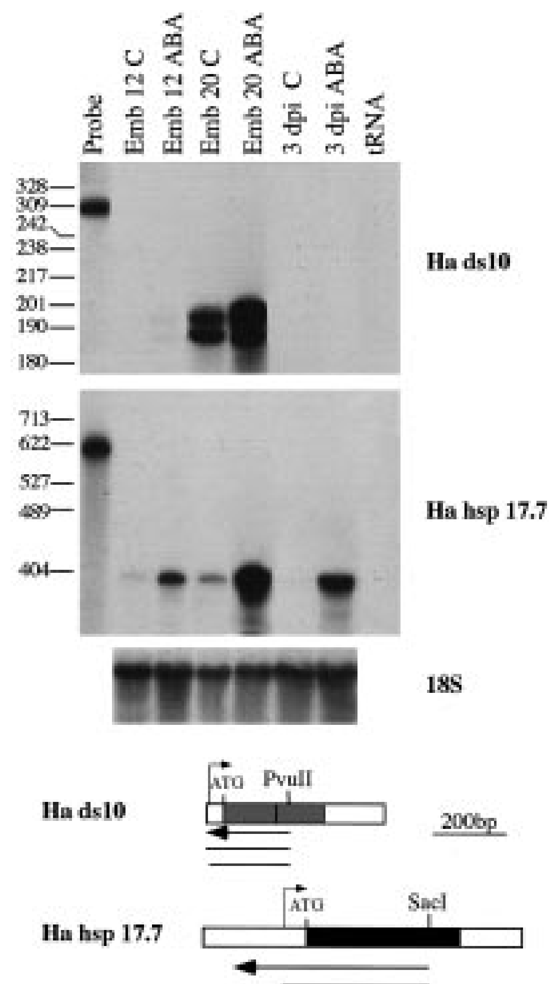

Figure 2. ABA response of *Ha ds10 G1*. Total RNA from control (C) or ABA-treated sample (ABA) was analyzed by RNAse A protection. Emb, embryos at 12 or 20 days post anthesis; 3 dpi, seedlings at 3 days after imbibition. The schemes at the bottom of the figure depict restriction maps of *Ha ds10* cDNA and *Ha hsp17.7 G4*, the probes used for the RNAse A protection assays, and a graphic interpretation for the protected RNA bands in each case. Coding sequences are shaded. As an internal control for ABA induction, we used hybridization with a *Ha hsp17.7 G4* riboprobe [6]. The ds10 riboprobe used in these assays was prepared from the cDNA plasmid [1], and it contained 30 nucleotides of 5′-UTR and 180 nucleotides of coding sequence. The largest protected ds10 fragment corresponds to mRNAs originating from transcription initiation usptream of site 1 (shown in the scheme). Size markers are from a mixture of *Hpa*II-digested pBR322 and pBluescript SK+ DNA.

group 1 *Lea* genes. The *ds10* intron differs however in its large size (1024 bp), something without precedents in genes belonging to the same family (Table 1). Large size introns is not a general feature of sunflower genes, as for example members from the 2S albumin (X06410), 11S albumin (M28832), and even other *Lea* (dehydrin, AJ002741) families, contain introns of only 79–190 bp.

Inspection of the *Ha ds10 G1* nucleotide sequence identified putative *cis*-acting regulatory elements (Figure 1C and data not shown). A potential TATA box is situated at −86 (upstream from the ATG). Despite our previous observation of homologous ds10 mRNA accumulation in response to either heat-stress or exogenous ABA application [1], we did not find in the proximal promoter region *cis* elements identical to those involved in the heat-shock and ABA responses. In the latter case we found two sequences with some similarity (1-nucleotide mismatch) with the consensus for the abscisic acid response element (ABRE) Em1a: a G-box element recognized by basic leucine zipper (bZIP) factors [17]. These putative G-boxes are located at −191 and −139 (Figure 1C and data not shown). However, both G-boxes also perfectly matched the consensus motif CACGTG, found in the promoters of dicot seed-storage proteins [4, 7]. Two other sequences perfectly matching the RY repeat (CATGCATG), an element involved in the seed-specific regulation of various plant genes [2], were found at −129 (RY-1) and −65 (RY-2), upstream and downstream respectively of a putative TATA-box (Figure 1C). The finding of the RY repeats increased our interest in investigating the regulation of *Ha ds10 G1*, as the presence of such elements, in promoters of the group 1 *Lea* gene family, was found to be characteristic of monocot rather than dicot plants, even allowing one single nucleotide mismatch [31].

The 5′-end of mRNAs from *Ha ds10 G1*, as well as their accumulation patterns in sunflower, were determined by a combination of primer extension and RNAse A protection strategies, as previously described for sunflower genes of the small heat-shock protein family [6]. The products of primer extension reactions were analyzed in sequencing gels, which identified potential 5′ ends of the *Ha ds10 G1* transcription unit (Figure 1A). Bands 1 and 2, at positions centered at −25 and −33 respectively, very likely represent functional transcription start sites, as they are located at appropriate distance from a putative TATA-box and reasonably match the results obtained with RNAse protection (Figure 1B, C). The 5′ end of the original cDNA (position −30 [1]) was only three nucleotides shorter that site 2. The interpretation of the third primer extension product (at −119, Figure 1A) is less concluding (see below). Similar primer extension results were obtained with RNA samples from 15 and 20 dpa embryos, indicating that the *Ha ds10 G1* promoter is active from stages of embryogenesis prior to seed desiccation (Figure 1A).

RNAse A protection experiments, performed using the same samples of total RNA from sunflower, confirmed the transcription initiation sites 1 and 2, and determined the accumulation patterns of mRNAs transcribed from *Ha ds10 G1* in seeds (Figure 1B). After hybridization of total RNA with the probe (Figure 1B, see Materials and methods), and full digestion with RNAse A, the size of protected RNA fragments is expected to indicate the distance from the 5′ mRNA ends to the 3′ end that is defined, in spliced mRNAs, by full protection of the first exon of *Ha ds10 G1* (see Figure 1B, bottom). Three protected mRNA fragments were observed from 10 dpa (Figure 1B), which in sunflower embryogenesis corresponds to a stage coincident with the peak of seed storage protein deposition [1]. The *Ha ds10 G1* mRNAs reached higher levels of accumulation at 18–20 dpa (coincident with seed desiccation). This accumulation slightly decreased in mature seeds (25–30 dpa).

The sizes of the three protected mRNA fragments were determined in longer sequencing gels (data not shown). Sizes for bands 1 and 2 (Figure 1B) were respectively of 162 and 173 nucleotides. These sizes reasonably match the primer extension results for initiation sites 1 an 2 (Figue 1A), after taking into account the mobility differences between DNA and RNA molecules of the same size in denaturing polyacrylamide gels [29]. Thus, the RNA protection experiments confirmed the presence of two close functional transcription initiation sites. The RNA polymerase does not appear to discriminate between these two initiation sites, as indicated by the similar abundance of mRNAs transcribed from them during zygotic embryogenesis (bands 1 and 2, Figure 1B). The interpretation of a third band (indicated with an asterisk in Figure 1B) was not conclusive. Its apparent size in sequencing gels (204 nucleotides) was too short to fit the third putative upstream initiation site observed by primer extension (Figure 1A, −119). Additional RNA protection experiments using another riboprobe (from the *Hpa*II site at position +1257 to the *Eco*RI site at position +1086, data not shown) determined that this band did not originate from protection of un-

Table 1. Intron in group 1 Lea genes.

Acc.[1]/Gene	Species	Position	Size (bp)	5′ splice site	3′ splice site
Z11158 / atem1	*A. thaliana*	118/119	185	GAAG/GTAT	TTAG/GAAG
Z11157 / atem6	*A. thaliana*	115/116	97	GAAG/GTCA	GAAG/GGAG
AJ002738 / BnEm6	*B. napus*	115/116	115	GAGG/GTCT	AAAG/GGAG
X60131 / emb-1	*D. carota*	115/116	100	GAAG/GTAC	TCAG/GGAG
X54518 / lea2	*G. hirsutum*	115/116	111	GAAG/GTGA	TTAG/GGAG
X13205 / d19	*G. hirsutum*	145/146	102	GAAG/GTAT	AAAG/GGAG
AJ224116 / **ds10**	*H. annuus*	145/146	**1024**	GAAG/GTAT	GAAG/GGCG
X78330* / b19.1	*H. vulgare*	118/119	119	GAAG/GTAC	GCAG/GGCG
X77157 / b19.1b	*H. vulgare*	119/120	99	GAAG/GTAC	GCAG/GGCG
X78332* / b19.3	*H. vulgare*	118/119	90	GAAG/GTAT	GCAG/GGCG
X78331* / b19.4	*H. vulgare*	118/119	87	GAAG/GTAT	GCAG/GGCG
U22102/osem	*O. sativa*	124/125	107	GAGG/GTAT	TCAG/GGCG
X73227 / emH2	*T. aestivum*	118/119	92	GAAG/GTAC	GCAG/GGCG
X73228 / emH5	*T. aestivum*	118/119	86	GACG/GTAT	GCAG/GGCG
X52103 / em	*T. aestivum*	122/123	71	GAAG/GTAC	GCAG/GGCG

[1]Accession numbers in the GeneBank-EMBL databases. *Accession numbers corresponding only to intron sequences. Positions of intron given as nucleotides after the predicted translation start codons.

spliced mRNA molecules. It could thus indicate the presence of a third 5′-distal initiation site (at about −75, Figure 1C, asterisk), which would be unique among group 1 *Lea* genes. Upstream of this site there is another potential TATA box at appropriate distance (Figure 1C, position −105). The third initiation site was not detected by primer extension, very likely because of mRNA secondary structure. The primer extension product at −119 would correspond to transcripts from one of the other highly homologous group 1 *Lea* genes present in sunflower [1].

ABA response of Ha ds10 G1

To investigate the possible regulation of *Ha ds10 G1* by ABA, we determined changes, in its mRNA accumulation, induced by exogenous ABA treatments (Figure 2). Total RNA from control or ABA-treated samples was analyzed by RNAse A protection. The effect of ABA was controlled by digestion, of the same RNA samples, after hybridization to a riboprobe from *Ha hsp17.7 G4*: a sunflower gene previously shown to be inducible by ABA in seedlings [6]. Total amounts of RNA in each sample were additionally controlled by northern hybridization to a 18S rRNA probe [1]. Experiments performed with excised embryos at two developmental stages (12 and 20 dpa) before and during seed desiccation, respectively, determined that *Ha hsp 17.7 G4* mRNAs substantially accumulated in response to ABA in either experimental condition (induction at least 10-fold). In contrast, we observed only a moderate ABA induction of *Ha ds10 G1* mRNAs in excised embryos (ca. 2–3-fold). The *ds10* mRNAs disappeared after germination by 3 days after imbibition (dpi). At this stage *Ha ds10 G1* mRNAs did not accumulate in response to ABA. In conclusion, compared to *Ha hsp17.7 G4*, *Ha ds10 G1* showed a small response to ABA only during embryogenesis. In embryos, this difference could be explained either by a reduced sensitivity to ABA of *Ha ds10 G1*, or (and) by distinct kinetics of the ABA response of both genes.

Changes in ds10 mRNA localization during zygotic embryogenesis in sunflower

As a first step to perform the *in situ* localization of ds10 mRNAs, we investigated the specificity of a RNA probe [ds10-3′(−)] that contained only part of the ds10 coding region and its 3′-flanking untranslated sequences (see Materials and methods). Previous Southern hybridization results, using the complete cDNA as a probe, detected 3–5 bands in sunflower genomic DNA digested with different restriction enzymes [1]. The RNA probe ds10-3′(−) detected only 1–2 bands in similar experiments (data not shown), which indicates that this probe hybridizes with a maximum of two very homologous genes.

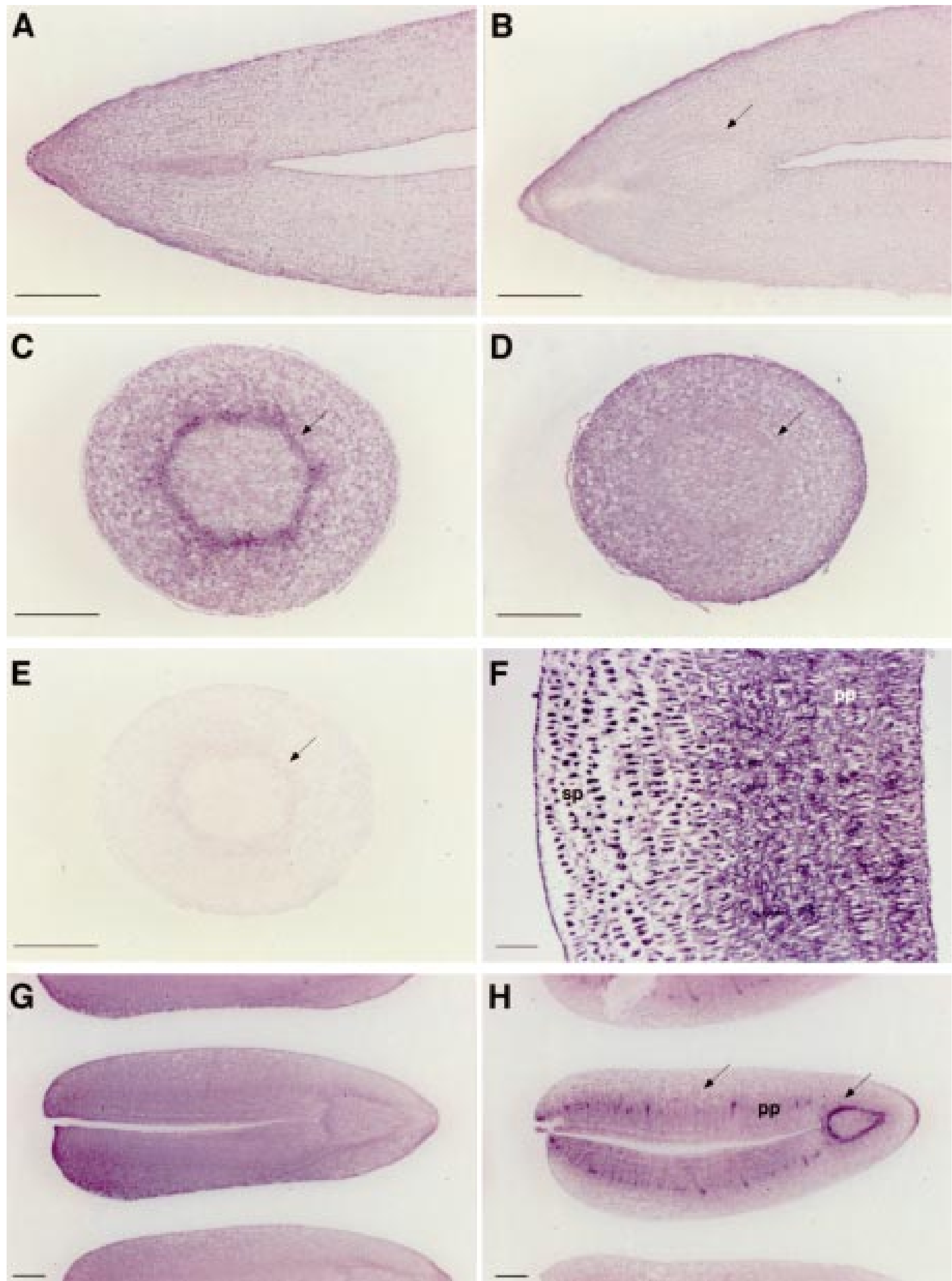

Figure 3. Localization of *Ha ds10 G1* mRNAs. Sections correspond to sunflower embryos at 12 dpa (panels A and B), 21 dpa (panels C–E) and 28 dpa (panels F–H). In each case, the following riboprobes were used for hybridization: A, C, F, H: ds10-3′(−); E: ds10-3′(+); B, D, G: 18S RNA. Scale bars = 500 μm (except in F = 125 μm). pp, palisade parenchyma; sp, spongy parenchyma. Arrows point to the procambium.

We tried to increase the probe specificity by performing stringent washings and RNAse A treatments after the *in situ* hybridization (see Materials and methods). These procedures should have resulted in detection of mostly *Ha ds10 G1* mRNAs. Even under these conditions, we cannot rule out marginal hybridization with highly homologous mRNA, and therefore we cautiously refer to the detected molecules as ds10 mRNA. *In situ* localization of *ds10* mRNAs were performed in sections from sunflower embryos using the ds10-3′(−) probe. As a negative control, we used the analogous sense strand probe ds10-3′(+), which should not hybridize to the ds10 mRNAs. Ds10 hybridization patterns were compared with those obtained with a riboprobe for 18S rRNAs. Results obtained are summarized in Figure 3. In agreement with our RNAse A protection results (Figure 1B), the ds10 mRNAs appear to accumulate to quite high levels from early stages of embryogenesis (12–15 dpa); as indicated by the short reaction times needed for the chromogenic reactions used to detect the RNA hybrids, i.e., ca. 2 h for both the ds10-3′(−) and 18S rRNA probes. At this stage their tissue distribution was quite homogeneous (i.e., comparable to that of 18S rRNA: Figure 3, panels A and B). At 21 dpa the homogeneous distribution of ds10 mRNAs started to show signs of some tissue specificity, with a marked localization in the vascular tissues of procambium (cf. Figure 3C and 3D). In embryos of 28 dpa, the distribution of ds10 mRNAs showed preferential localization in some cells of the cotyledons, as well as in the procambium (Figure 3H). Inspection at higher magnification demonstrated that, at this stage, the detected mRNAs were preferentially localized in the palisade parenchyma cells of cotyledons, with also a possible difference in its intracellular distribution compared to that in the spongy parenchyma (Figure 3F). This localization contrasted with the homogenous distribution of total RNA, also observed at 28 dpa with the 18S rRNA probe (Figure 3G). Control hybridizations with the probe ds10-3′(+) did not reveal artifactual hybridization signals (Figure 3E). In summary, we observed a homogeneous and abundant localization of ds10 mRNAs in embryos from 12–20 dpa, which evolved later in seed maturation, to a tissue specificity that is unique among plant genes belonging to the group 1 *Lea* family.

Transcriptional activation of the Ha ds10 G1 *promoter by ABI3 in sunflower embryos*

We have assayed *trans*-activation by the *Arabidopsis* ABI3 factor [15] of two reporter plasmids incorporating the *Ha ds10 G1* promoter (Figure 4). This experimental design has produced results indicating the role of ABI3-like factor(s) in the transcriptional activation of the *Ha ds10 G1* promoter. Five independent experiments have been performed, and each combination of reporter and reference (± effector) plasmids has been bombarded 5 times in each experiment (for details, see Materials and methods). We observed a clear *trans*-activation of the *Ha ds10 G1* promoter by ABI3 (46.2-fold). Deletion of the RY-1 repeat reduced this activation (to 26.3-fold) without abolishing it (Figure 4). The observed induction by ABI3 using the reporter plasmid F2, and the reduction of this induction using F2Δ, were statistically significant (respectively: $F = 161.695$, $P = 0.001$; $F = 17.529$, $P = 0.001$), as determined by ANOVA analyses performed after normalization of the experimental data using procedures previously described [6]. The RY-1 repeat does not appear to be sufficient for the ABI3 response observed in the context of construct pSKds10F2. RY-1 might thus function in concert with other *cis* elements of the *Ha ds10 G1* promoter, perhaps including the RY-2 repeat (Figure 1C). However, these results did not formally demonstrate a functional involvement of the RY-1 repeat in the transcriptional regulation of the *Ha ds10 G1* promoter, as the internal deletion in pSKds10F2ΔRY1 might also have altered the relative positions of other *cis* elements.

Discussion

We established that the *Ha ds10 G1* gene has unusual characteristics among other described members of the group 1 *Lea* family in plants. We observed both structural and gene expression regulation peculiarities that indicate that *Ha ds10 G1* is a divergent member of this gene family. *Ha ds10 G1* might have evolved to be expressed, and to function, in specialized seed tissues.

Excluding the wheat Em gene (see review in [9] and references therein), the expression of the group 1 *Lea* (*Lea*-1) genes is restricted to embryo tissues; either in non-stress conditions, or in response to exogenous ABA treatments or stress conditions. In seedlings, these treatments induce the expression of some of the *Lea*-1 genes, but usually at much lower

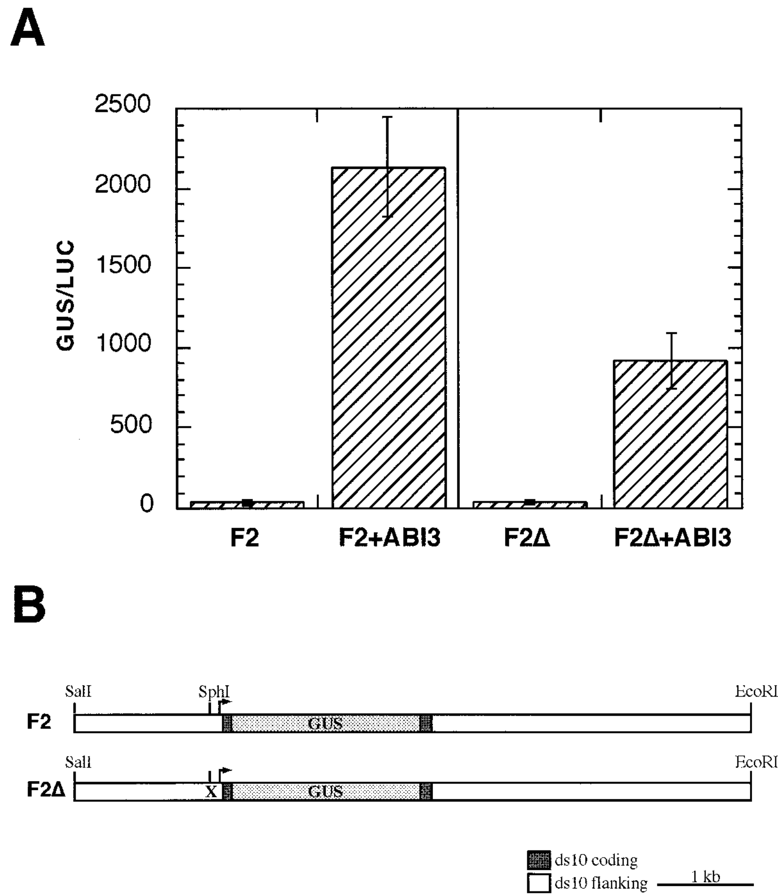

Figure 4. Transient *trans*-activation of the *Ha ds10 G1* promoter in sunflower embryos: effects of ABI3 and of internal deletion of the RY1-repeat. A. Bars represent mean β-glucuronidase (GUS) activity, normalized to luciferase activity (LUC). The reporter plasmid used in each combination is indicated: F2, pSKds10F2; F2Δ, pSKds10F2ΔRY1. Samples labelled +ABI3 included the effector plasmid p35S::ABI3 (for details, see Materials and methods). Standard errors of the means are indicated ($n = 25$). B. Maps of the reporter plasmids. The position of the RY-1 deletion is marked by X.

levels than observed in seeds. This limited induction has been considered a remnant from embryo regulation: i.e., it would be mediated by embryo-specific factors that are diluted and disappear shortly after germination. In dicot plants *Lea*-1 gene expression is absent from vegetative tissues formed after seed germination (reviewed in [9]). We demonstrated, by RNAse A protection experiments, that the *Ha ds10 G1* mRNAs accumulated in a strictly embryo-specific manner: without being responsive to ABA or water stress in 3 dpi seedlings, or at later stages after germination (Figures 1, 2 and data not shown). Additional experiments, performed with excised immature embryos at two embryogenesis stages, confirmed the lack of a substantial ABA response (Figure 2). These characteristics have been confirmed by other analyses with transgenic tobacco plants containing ds10::GUS chimeric genes (Prieto-Dapena *et al.*, in preparation).

The temporal expression patterns of *Ha ds10 G1* mRNAs during zygotic embryogenesis and its putative regulation have features in common, but also important differences, with those of other plant *Lea*-1 genes. The observed mRNA accumulation from a stage that precedes seed-desiccation (10 dpa, Figure 1B) would be similar for example to that of the *Arabidopsis* Em1 [14], carrot Emb-1 [34], and maize Emb564 [33] genes. In contrast, other *Lea*-1 genes such as *Arabidopsis* Em6 [14] are expressed only during later stages of seed desiccation. The 'early' expression of some *Lea*-1 genes would be comparable to that of *Lea*-A genes of cotton. This is a subset of *Lea* genes, from different groups, that show mRNA accumulation peaks in coincidence with the

rise of ABA, before desiccation, in the developing embryo [19]. Additional gene expression analyses in embryo mutant backgrounds determined that ABI3, a protein involved in the regulation of seed-specific expression programs, is required for the efficient expression of the Em1 and Em6 genes in *Arabidopsis*. Accumulation of the Em1 and Em6 mRNAs was also dependent on endogenous ABA, as it decreased in ABA-deficient mutants [26]. Experiments with other ABA-deficient and insensitive mutants established that ABA and Vp1, a regulatory gene of maize homologous to ABI3, control the expression of the wheat Em, and homologous maize gene(s). In that case, additional transient expression experiments in maize embryos demonstrated a transcriptional activation of the wheat Em promoter, that was mediated by Vp1 [23]. The results depicted in Figure 4 represent similar evidence, obtained to our knowledge for the first time with a promoter from a dicot *Lea*-1 gene: ABI3 and PvAlf (data not shown), a homologous transcription factor recently cloned from *Phaseolus vulgaris* [3], *trans*-activated the *Ha ds10 G1* promoter in sunflower embryos. These results, combined with the previously discussed limited ABA response, suggest that the *Ha ds10 G1* promoter might be regulated during embryo maturation by mechanisms that would differ, in the relative contribution of ABA, from those proposed for the *Arabidopsis* and wheat Em genes. In the case of *Ha ds10 G1*, ABI3-like factors are likely to be involved, but most likely mainly through ABA-independent pathways. Other *Lea*-1 genes, such as Em1 and Em6, would be controlled by ABI3 through both ABA-dependent and -independent pathways [26]. We would like to add some caution to this suggestion as the effects of exogenous ABA on gene expression do not always correlate with those of endogenous ABA content [16]. We also cannot rule out a higher ABA response of *Ha ds10 G1* under different experimental conditions (i.e., with other incubation times of younger embryos). *Ha ds10 G1* also differs from other dicot *Lea*-1 in the presence of perfect-consensus, and perhaps functional, RY repeats (Figures 1C and 4). This is something more characteristic of *Lea*-1 genes from monocots, as first suggested by Stacy *et al.* [31], and confirmed to date by sequence analyses of the genes listed in Table 1 (data not shown). In addition to RY-1, other putative *cis*-acting elements present in the 5′-flanking sequences of *Ha ds10 G1*, and elsewhere in this gene, might be involved in its regulation. The unusually long intron, found at a conserved position, is perhaps the most extraordinary structural feature of the *Ha ds10 G1* gene (Figure 1B, bottom, and Table 1). Preliminary results indicate that this intron could contribute to the seed-specific regulation of *Ha ds10 G1* because, in transgenic plants with chimeric ds10::GUS genes, the presence of the intron reduced expression in pollen, the only non-seed tissue where these genes showed some activity (Prieto-Dapena *et al.*, in preparation). In summary, we conclude that the evolutionary divergence of *Ha ds10 G1*, first noticed by comparison of coding and mRNA flanking sequences [31], also extends to other features of its gene structure and regulation.

The spatial expression patterns of ds10 mRNAs were also singular: the homogeneous distribution of ds10 mRNAs in immature embryos (Figure 3A), and their preferential localization in the palisade parenchyma cells of cotyledons, and in the procambium, later in embryogenesis (Figure 3C, F and H) differ from other homologous plant genes, for which in the literature there are scarce comparable studies. Thus, the carrot Emb-1 mRNAs accumulated in both somatic and zygotic embryogenesis throughout globular embryos, but, as embryogenesis continued, they preferentially localized only to the meristematic regions, particularly the procambium [34]. In other studies performed in dry seeds, the *Arabidopsis* Em1 mRNAs showed preferential accumulation in the provascular bundle of cotyledons and embryo axis, as well as in the central parenchyma, epidermis, and outer layers of the cortex in the embryo axis. Changes in localization at different stages of embryogenesis were not studied in this case [14]. Our results, as in precedent works include only mRNA localization analyses. For functional hypotheses one must assume that mRNA accumulation and localization would indicate that of the encoded *Lea* protein (i.e., that there is little effect of translational regulation). This assumption has been supported so far in the few studies were both *Lea* protein, and mRNA accumulation, have been determined (reviewed in [11] and [9]). The observation of non-uniform mRNA localization patterns for *Lea* genes belonging different groups, including *Lea*-1 [34] has set forward the hypothesis of a possible functional specialization of the proteins belonging to each group. This hypothesis could complement the more general hypotheses based in the structural features characteristic of each group (reviewed in [12]), and in the homogeneous distribution and abundance of some *Lea* proteins [28]. In the case of *Lea*-1 proteins, the proposed function of water-binding and dehydration protection, could be expanded to include specific

targets (molecules or tissues) for different proteins. During desiccation the ds10 protein could perform its functions in tissues as the procambium (similar to suggested for other *Lea*-1 proteins), but most interestingly in the palisade parenchyma, a cotyledon tissue specialized in the deposition of reserve substances such as storage oil and proteins. Reserve oil and protein are stored in specialized sub-cellular organelles (oil bodies and protein bodies) that must endure embryo desiccation. Another possible association during desiccation, between protein bodies and *Lea* proteins, has been previously observed for DC8, a carrot member from group 3 [13].

The detailed regulatory mechanisms of *Ha ds10 G1* and the specific functions of the encoded protein remain to be determined. The results reported here confirm their evolutionary divergence from other plant *Lea*-1 proteins. We also identified specific tissues where this protein could be expressed. The seed-specific expression patterns of *Ha ds10 G1* could have potential interest for applied research targeted to seed protein or lipid modification.

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