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RESEARCH ARTICLE

Developmental and Hormonal Regulation of Sunflower Helianthinin Genes: Proximal Promoter Sequences Confer Regionalized Seed Expression

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DNA elements involved in the regulation of two sunflower helianthinin genes were identified by analysis of β-glucuronidase (GUS) expression in transgenic tobacco driven by sequences derived from the 5′ upstream regions of these genes. A 2.4-kb upstream region of the helianthinin gene *Ha*G3-A conferred rigorous developmental GUS expression in transgenic tobacco seeds with no significant GUS activity in nonembryonic tissues. Regions of the helianthinin upstream regulatory ensemble (URE) conferred ectopic expression in nonembryonic tissues when analyzed outside of the context of the complete helianthinin regulatory complex. A proximal promoter region was identified that conferred significant GUS expression in seeds but not in leaves of transgenic tobacco. Three sequence motifs that bind to seed nuclear proteins were identified in the proximal promoter region; mutations in these motifs significantly reduced the level of nuclear protein binding. Another important class of *cis*-regulatory elements was identified in the helianthinin URE that conferred abscisic acid-responsive GUS expression. In the full-length helianthinin URE, these elements only responded to abscisic acid in the developing seed, suggesting that the helianthinin gene contains additional regulatory elements, possibly in the proximal promoter region, that ensure hierarchical control in the developing seed.

INTRODUCTION

Seed development involves embryogenesis and maturation events as well as physiological adaptation processes that occur within the seed to ensure progeny survival. Developing plant seeds accumulate and store carbohydrate, lipid, and protein that are subsequently used during germination. Expression of storage protein genes in seeds occurs primarily in the embryonic axis and cotyledons and in the endosperm of developing seeds but never in mature vegetative tissues. The expression patterns of seed proteins are highly regulated, both spatially and temporally, during seed development (Goldberg et al., 1989; Pereze-Grau and Goldberg, 1989; Guerche et al., 1990). Furthermore, storage proteins are frequently processed and in many cases are targeted to protein bodies (Shotwell and Larkins, 1988). As a consequence of the rigorous developmental expression of seed protein genes, the structure and expression of these genes from numerous plant species have been analyzed extensively (Goldberg et al., 1989; Perez-Grau and Goldberg, 1989; Thomas, 1993). Significant progress has been made in the elucidation of *cis*-acting DNA sequences involved in the regulated expression of these genes (reviewed in Thomas, 1993).

Abscisic acid (ABA) is involved in numerous physiological responses of the plant. These mediate adaptation processes to environmental stresses; such as water deficit, salt stress (reviewed in Skriver and Mundy, 1990), and in some cases mechanical stress (Peña-Cortés et al., 1989). During embryogenesis and seed development, abscisic acid (ABA) functions by preventing precocious germination before desiccation and by promoting embryo maturation and developmental arrest during dormancy (reviewed in Quatrano, 1987). ABA apparently is involved in regulating expression of specific genes in embryos of cotton, rape, soybean, sunflower, wheat, rice, barley, and maize (reviewed in Quatrano, 1987; Skriver and Mundy, 1990; Thomas et al., 1991). For example, ABA is required for the accumulation of the 12S seed protein cruciferin in *Brassica* embryos (Finkelstein et al., 1985) and for the continued synthesis of the β subunit of β-conglycinin in soybean cotyledons (Bray and Beachy, 1985). In maize, the initiation of synthesis and accumulation of storage globulins is ABA dependent (Rivin and Grudt, 1991).

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We are interested in factors controlling the expression of sunflower genes encoding the 11S seed protein helianthinin. Like other seed protein genes, sunflower helianthinin genes are expressed exclusively in the developing seed (Bogue et al., 1990). We identified *cis*-regulatory elements contained within helianthinin upstream regulatory ensembles (UREs) by analysis of β-glucuronidase (GUS) reporter gene expression in transgenic tobacco. Expression was driven by sequences derived from the UREs of helianthinin genes *Ha*G3-A and *Ha*G3-D. A 2.4-kb upstream region of the helianthinin gene *Ha*G3-A confers GUS expression in transgenic tobacco seeds with no detectable GUS activity in nonembryonic tissues (Bogue et al., 1990). Elements of the helianthinin URE conferred ectopic expression when analyzed outside the context of the complete sunflower regulatory complex; these elements include an important class of *cis*-acting DNA sequences that confer ABA-responsive GUS expression. In the full-length (FL) helianthinin URE, these elements only responded to ABA in the developing seed, suggesting that additional regulatory elements are present that ensure hierarchical control during seed development. Furthermore, we found that a proximal promoter region of both the helianthinin *Ha*G3-A and *Ha*G3-D genes binds multiple nuclear proteins and confers significant seed-specific GUS expression in transgenic tobacco.

RESULTS

Helianthinin Promoter Elements Direct Ectopic GUS Expression in Tobacco

Previously, we showed that the region (−2376 to +24) upstream of the sunflower helianthinin gene *Ha*G3-A conferred rigorous, seed-specific expression of the GUS reporter gene (Bogue et al., 1990). The upstream regions of sunflower helianthinin genes *Ha*G3-A and *Ha*G3-D contain extensive regions of similarity (Bogue et al., 1990), as illustrated in Figure 1. For example, the *Ha*G3-D helianthinin gene contains a 404-bp region that is 98% similar to a sequence present in the *Ha*G3-A upstream region with the 5′ end at the SalI site (−1527). AT-rich DNA sequences that interact with proteins from sunflower embryo and hypocotyl nuclear extracts were previously identified in both upstream regions (Jordano et al., 1989) and are shown in Figure 1. Subsequent experiments demonstrated high mobility group (HMG) or HMG-like proteins probably bound these AT-rich motifs (J. Jordano and T. L. Thomas, unpublished results).

Initially, expression of chimeric GUS reporter genes in transgenic tobacco was examined to identify potential *cis*-regulatory elements; these chimeric genes included overlapping fragments of the upstream regions of the helianthinin genes (Figure 1). *Ha*G3-A-FL and *Ha*G3-A-S-Δ1 are transcriptional GUS fusions beginning with +24 of the *Ha*G3-A URE and the promoterless GUS reporter gene pBI101.1 (Jefferson et al., 1987). All other helianthinin GUS reporter genes contain chimeric promoters

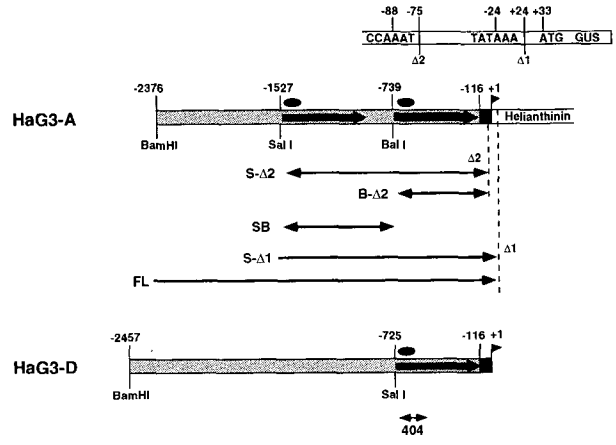

Figure 1. Fragments of *Ha*G3-A and *Ha*G3-D Used for GUS Constructions.

Maps of the upstream regions of *Ha*G3-A and *Ha*G3-D are shown. The regions between −116 and +1 are identical and are indicated by the black-filled regions. The bold arrows within these sequences indicate large duplicated regions that each contain a nuclear protein binding site (black-filled oval; Jordano et al., 1989). Exonuclease III digestions generated two *Ha*G3-A deletions shown at the top: Δ1 contains *Ha*G3-A TATA and CAAT boxes, whereas Δ2 contains only the CAAT box. The fragments were ligated into pBI101.1 (Δ1 fragments) or pBI120 (Δ2 and other fragments). Constructions were made in the orientations indicated by the arrows. The junction of each construction was sequenced to confirm orientation. These fragments and subsequent GUS constructions are named according to their end sites as shown to the left of each fragment. S, SalI; B, BalI. The construction containing the *Ha*G3-A FL regulatory region (Bogue et al., 1990) as well as the *Ha*G3-D constructions (Jordano et al., 1989) were described previously.

derived from the *Ha*G3-A or *Ha*G3-D upstream regions and the truncated cauliflower mosaic virus (CaMV) 35S promoter (−90 to +8), termed ΔCaMV (Jordano et al., 1989). Results obtained with seeds, leaves, and roots are included in Table 1. All constructions containing some portion of the UREs of helianthinin genes *Ha*G3-A and *Ha*G3-D conferred GUS activity in transgenic tobacco seeds. It should be noted that all plants analyzed in Table 1 contained a single segregating kanamycin resistance (*kan*^{r}) locus. Constructions including the helianthinin proximal promoter elements and a minimum of 1.5 kb of the *Ha*G3-A URE (FL and S-Δ1) demonstrated rigorous tissue-specific GUS expression with no detectable GUS activity in any tissues of transgenic seedlings. However, the FL construct was expressed in mature seeds at approximately fivefold higher levels compared to S-Δ1. Most constructions other than FL and S-Δ1 demonstrated significant ectopic expression in roots of transgenic seedlings and in leaves in several cases. It is noteworthy that rigorous seed-specific expression was obtained only with constructs FL and S-Δ1 that include the proximal promoter upstream regions between −75 and +24. No GUS activity was found in tissues of seedlings

Table 1. Relative GUS Expression Driven by Elements of Helianthinin UREs in Embryonic and Vegetative Tissues of Transgenic Tobacco[a]

Construct	Relative GUS Activity (%)[b]			ABA
	Seed	Leaf	Root	Responsive[c]
*Ha*G3-A				
FL (−2376 to +24)	100	0	0	+
S-Δ1 (−1527 to +24)	18	0	0	ND
S-Δ2 (−1527 to −75)				
F	190	0	111	ND
R	111	2.6	205	+
B-Δ2 (−739 to −75)				
F	58	11	42	+
R	38	22	21	+
SB (−1527 to −739)				
F	58	0	74	−
R	63	1.6	480	+
*Ha*G3-D				
404 (−725 to −322)				
F	48	0.5	36	+
R	48	11	68	+
Controls				
pBI101	0	0	0	−
pBI120	0	0	0	−

[a] Mature (30 DPF) seeds and seedlings (18 to 20 DPI) of transgenic tobacco containing chimeric helianthinin–GUS constructions (Figure 1) were assayed for GUS activity. Forward (F) and Reverse (R) refer to the orientation of each helianthinin fragment with respect to the truncated 35S CaMV promoter of pBI120 (refer to text and Figure 1).
[b] GUS activity levels were determined from three to 10 transgenic plants for each construction. The average for each construction was normalized to that of *Ha*G3-A-FL in mature seeds. In this series of experiments, the average GUS expression for *Ha*G3-A-FL was 825 ± 51 pmol of 4-methylumbelliferone per milligram per minute.
[c] *Ha*G3-A-FL is only ABA responsive in developing seeds 12 to 18 DPF (see Table 2 and Figure 6). For all others, ABA response was predicted from GUS expression in desiccated leaves and subsequent demonstration that seedlings respond directly to exogenous ABA. +, more than threefold induction of GUS activity above background; −, indistinguishable from background; ND, not determined.

and in seeds containing either ΔCaMV–GUS (pBI120) or the GUS reporter with no promoter (pBI101).

Roots of seedlings containing the S-Δ2 and SB constructions in the reverse orientation (S-Δ2/R and SB/R, respectively) showed levels of GUS activity two- to fivefold greater than FL in seeds; these levels were four- to eightfold greater than that observed with the truncated CaMV 35S promoter driving GUS expression in roots of transgenic tobacco (data not shown). A total of five independently transformed plants containing the SB/R construction were assayed yielding the results in Table 1. Thus, this high SB/R-driven GUS expression in roots does not appear to be due to local or global position effects. Overall, SB/R plants expressed GUS primarily in roots of seedlings 18 days postgermination and mature plants with minimal activity in leaves or other mature plant tissues (data not shown).

GUS expression profiles in developing seeds of transgenic tobacco containing a series of 5′ deletions of *Ha*G3-A-FL are shown in Figure 2. GUS activity was determined in leaves and in staged seeds of transgenic plants containing the indicated deletions; a subset of these data are presented in Table 2 to facilitate quantitative comparisons. Deletions had varied effects on GUS expression (Figure 2B; Table 2). Deletion of FL to −74 resulted in an ~20-fold decrease in the level of GUS expression in mature seeds, but tissue specificity (i.e., seed specificity) was retained. For example, the ratios of GUS expression in seeds 16 days postflowering (DPF) and in leaves for the −116 and −74 deletions were 21 and 22, respectively. However, temporal regulation was less rigorous; at 8 DPF, the −74 deletion expressed GUS at the maximal level compared to the levels observed at 16 and 30 DPF (Table 2). Furthermore, deletions to −651 or −739 resulted in substantially higher levels of expression at 16 DPF compared to FL or the −1527 deletion (Figure 2B; Table 2). Levels of GUS expression driven by the −739 and −651 deletions decreased significantly and reproducibly in mature seeds (30 DPF). The variations in GUS expression between FL, −1527, and −739 at 16 and 30 DPF suggest the presence of potent positive and negative "very distal" *cis*-regulatory sequences.

Helianthinin Proximal Promoter Region Confers Regionalized GUS Expression in Seeds

The proximal promoter regions (PPRs) of helianthinin genes *Ha*G3-A and *Ha*G3-D are the only regions of sequence identity in the UREs of these two nonallelic genes (Bogue et al., 1990). Disruption of PPR at −75, resulted in ectopic GUS expression (Table 1). Furthermore, deletion of the helianthinin promoter to −116, and subsequently to −74, resulted in significant reduction of GUS expression in seeds but retention of seed-specific expression (Figure 2). Histochemical analysis of mature transgenic tobacco seeds containing deletions described in Figure 2 revealed regionalized GUS expression driven by the helianthinin PPR (Figures 3B, 3C, and 3D). Deletions to −74 resulted in GUS activity in the cotyledons extending through the shoot apical region but not into the root apical region (Figure 3C). Additional sequences to −116 did not alter the shoot/cotyledon pattern. However, sequences between −116 and −321 extended the tissue range of GUS expression to include most of the root apical region (data not shown). Staining of cotyledons, shoot, and the entire root apical region was obtained when the −739 deletion was examined (Figure 3A).

The preceding results suggested that the helianthinin PPR is critical to seed-specific expression. We tested this hypothesis directly by transferring a PPR–GUS fusion to tobacco (Figure 4). A synthetic PPR (−116 to +24) was fused to a chimeric GUS reporter gene that included a cap-independent translational enhancer (Figure 4A); the latter element represents

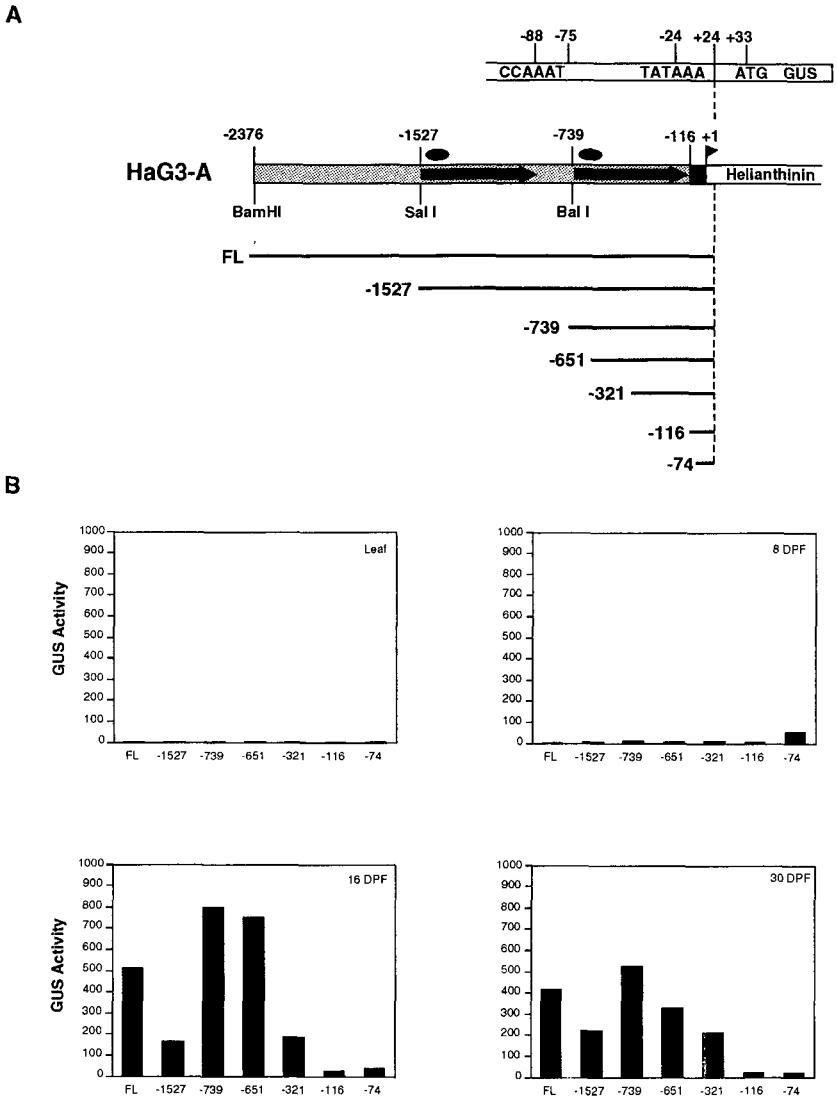

Figure 2. Deletion Analysis of Helianthinin Upstream Regulatory Ensemble.

(A) Helianthinin *HaG3*-A URE indicating 5′ promoter deletions fused to the GUS reporter gene. In all cases, the 3′ terminal nucleotide of the helianthinin URE (+24) is fused out of frame with the GUS reporter (indicated above the map). Symbols are as given in the legend to Figure 1. **(B)** GUS expression in developing seeds and mature leaves of transgenic tobacco containing deletions indicated in **(A)**. GUS activity is in picomoles of 4-methylumbelliferone per milligram per minute.

nucleotides 12 to 144 of the nontranslated region of the tobacco etch virus (TEV)–positive strand RNA (Carrington and Freed, 1990). The TEV translational enhancer was included because of limited GUS expression driven by the helianthinin PPR (Figures 2B and 3B). It was shown previously that the TEV enhancer increased efficiency of mRNA translation by as much as 10-fold in some cases (Carrington and Freed, 1990) and that its function appears to be sequence and tissue independent (Carrington et al., 1991).

A total of six independently transformed transgenic tobacco were generated containing PPR–GUS; significant GUS activity was detected in developing seeds of all R_0 plants with little if any detectable GUS activity in nonembryonic tissues of mature plants. Progeny of three plants were analyzed in greater detail. Figure 4B shows that the helianthinin PPR drives significant GUS expression in developing seeds of transgenic tobacco; the level of TEV-enhanced PPR-driven GUS activity in mature seeds was equivalent to or greater than that obtained

with the full-length helianthinin promoter. PPR-driven GUS expression in leaves was negligible and comparable to that observed for the full-length helianthinin promoter (Bogue et al., 1990). Histochemical localization of GUS activity in mature seeds containing PPR–GUS (Figures 3D and 3E) revealed a pattern similar to that observed with −116 and −74 deletions (Figures 3B and 3C) with intense staining over the embryonic shoot apex extending into the cotyledons but not extending into the region including the embryonic root apex. The presence of the TEV translational enhancer expanded the dynamic range of GUS expression driven by the helianthinin PPR but did not appear to significantly perturb the spatial expression pattern of PPR–GUS. As a consequence, use of the TEV enhancer for other weak or basal promoters may prove useful for detailed analysis.

Multiple Nuclear Proteins Bind to Sequences in the Helianthinin PPR

We investigated the interaction of sunflower nuclear proteins with helianthinin proximal promoter sequences using gel mobility shift assays, competitive gel mobility shift assays, and DNase I protection experiments (Figure 5). A radiolabeled *Ha*G3-A fragment (−135 to +24), used as a probe in DNase I footprint experiments, detected five regions that significantly interact with sunflower embryo nuclear proteins (Figures 5A and 5D); additional, weaker interactions were also observed. Protected sequences are summarized in Figure 5E; they include an AGATGT motif at −111 and −58, TGATCT at −83 and −41, and the CCAAAT motif at −91.

We further investigated the interaction of sunflower nuclear proteins with the AGATGT motifs using gel mobility shift assays. Mutations (M1; Figure 5E) in the distal AGATGT motif (−111) dramatically decreased the ability of sunflower nuclear proteins binding to this region (Figure 5B; cf. lanes 2, 3, 6, and 7 with lanes 5 and 8). Sunflower nuclear proteins from developing seeds form additional DNA–protein complexes (C1 to C3) compared to extracts from the hypocotyl, a tissue in which the helianthinin gene is not expressed. Results of gel mobility shift experiments resolved by PAGE for longer times revealed that complex C1 included two or more bands with embryo nuclear proteins, but only one band when hypocotyl nuclear proteins were used (data not shown). The additional DNA–protein complex formed with sunflower embryo nuclear proteins may play an important role in the tissue-specific expression of helianthinin genes.

Because the helianthinin PPR region (−116 to +24) contains two identical AGATGT motifs, we investigated if the same sunflower embryo nuclear protein(s) binds both motifs by performing competition gel mobility shift assays (Figure 5C). The interaction of labeled probe including the distal AGATGT motif (−116 to −91) with sunflower embryo nuclear extracts was studied in the presence of various competitors. The probe sequence itself (−116 to −91) competed effectively under these conditions, but a sequence (−116 to −91) containing the M1 mutant motif (gcGAcGT) failed to compete effectively (Figure 5C, cf. lanes 3 and 4). The proximal AGATGT motif, which is included in the −88 to +24 sequence, competed as effectively, or more so, as the distal AGATGT motif (Figure 5C, lanes 3 and 5). The results suggested that the protein(s) interacts with both proximal and distal AGATGT motifs.

We also studied the interaction of sunflower embryo nuclear protein(s) with the CCAAAT or Y-box motif (−91) by comparing the binding activity of the nuclear protein(s) with the wild-type and mutant CCAAAT motifs (M2; Figure 5E) in a DNase I footprint experiment (Figure 5D). We found the mutant motif (CCAAgT) abolished the binding activity of the protein(s) (Figure 5B, cf. lanes 4 and 5 with lanes 7 and 8) when similar specific activity probes were used.

Table 2. The Helianthinin PPR Directs Seed-Specific GUS Expression[a]

	GUS Activity (pmol 4-MU/mg/min)			
Construct	Leaf	8 DPF	16 DPF	30 DPF
FL	1.2 ± 0.95	3.4 ± 2.8	514 ± 120	415 ± 220
	(1)	(2.8)	(428)	(345)
−739	2.3 ± 0.74	14.4 ± 5.8	797 ± 300	527 ± 138
	(1)	(6.3)	(347)	(230)
−116	1.3 ± 1.0	7.0 ± 0.95	27 ± 11	21 ± 9.6
	(1)	(5.4)	(21)	(16)
−74	1.8 ± 0.52	54.0 ± 8.3	39 ± 11	23 ± 11
	(1)	(30)	(22)	(13)

[a] Subset of data from Figure 2. Values in parentheses are the ratios of GUS expression in developing seed to those in leaf of transgenic tobacco containing the same construction. GUS activity is given in picomoles of 4-methylumbelliferone per milligram per minute.

Elements of Helianthinin Promoter Regions Respond to ABA

Analysis of GUS fusions containing discrete elements of the *Ha*G3-A and *Ha*G3-D UREs identified two regions containing ABA-responsive *cis*-regulatory elements (ABREs) in the URE of *Ha*G3-A and one ABRE-containing region in *Ha*G3-D (Table 1). Jordano et al. (1989) cloned a region containing the only functionally identified ABRE from *Ha*G3-D (Figure 1) into a GUS cassette and transferred it into tobacco. We found that this construct, called *Ha*G3-D-404, directed ectopic GUS expression in leaves of transgenic tobacco in response to desiccation and exogenous ABA (Table 1).

We examined the expression of *Ha*G3-D-404 and *Ha*G3-A-FL in developing tobacco seeds and found that *Ha*G3-D-404 exhibited ectopic expression in developing tobacco seeds when compared to expression of *Ha*G3-A-FL (Figure 6). The results represented two distinct developmental profiles based on the time of initial appearance of GUS activity in developing

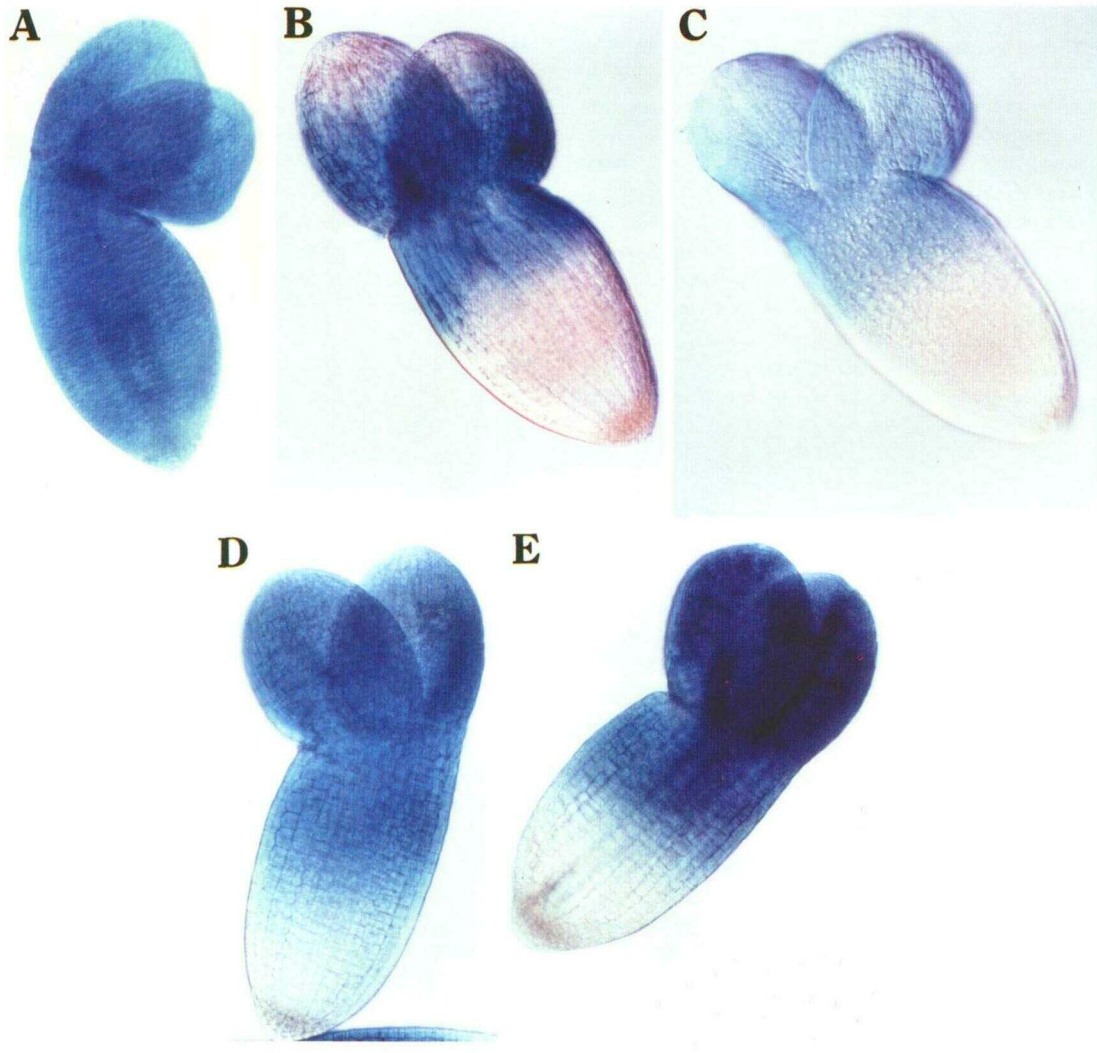

Figure 3. Histochemical Analysis of GUS Expression in Seeds of Transgenic Tobacco Containing Helianthinin URE–GUS Fusions.

(A) to **(C)** X-gluc staining of transgenic tobacco seeds containing selected 5′ deletion GUS fusions. **(A)** contains −739; **(B)** −116; **(C)** −74. **(D)** and **(E)** X-gluc staining of transgenic tobacco seeds containing the helianthinin PPR (−116 to +24) and TEV-enhanced GUS fusions. **(D)** contains PPR–GUS-3; **(E)** PPR–GUS-6 (see Figure 4 for additional details).

embryos and the qualitative and quantitative characteristics of the resulting expression patterns. As shown previously (Bogue et al., 1990), the full-length helianthinin URE (*Ha*G3-A-FL) shows correct temporal regulation where accumulation of GUS begins 12 DPF. It is important to note that 12 DPF is the earliest time tobacco expresses detectable levels of seed protein (Chen et al., 1988). In contrast, ectopic *Ha*G3-D-404 driven GUS expression occurs by 4 DPF and reaches a peak at ~10 to 12 DPF followed by a rapid decline to less than one-third of the maximal level at 30 DPF. Expression of *Ha*G3-D-404-GUS correlated with elevated levels of ABA during seed development preceding the maturation and desiccation phase of seed development. As expected, *Ha*G3-A-FL expression appears to reflect these later programs.

Results in Table 1 and Figure 6 indicated that the −725 to −322 region of *Ha*G3-D contained an ABRE. The ABRE within *Ha*G3-D-404 responded to endogenous and exogenous ABA in other vegetative tissues, including seedlings. Transgenic tobacco seedlings (21 days postimbibition [DPI]) containing *Ha*G3-D-404 and *Ha*G3-A-FL were transferred to media containing 0 to 10 mM ABA, and GUS activity was subsequently determined (Table 3). Seedlings containing *Ha*G3-D-404 were ABA inducible by day 1 at all ABA concentrations. Maximum induction, exceeding 200-fold, occurred after 3 days of ABA exposure at concentrations of 10 mM, but significant induction of 19- and 70-fold occurred on day 3 at 0.1 and 1.0 mM ABA, respectively. Induction of *Ha*G3-A-FL in parallel experiments was insignificant.

Additional Promoter Sequences Control Helianthinin ABREs

The expression of many, but not all, seed protein genes can be modulated in embryos in vitro by the application of exogenous ABA (see Introduction), and it is probable that ABA plays a significant role in modulating seed storage protein gene expression during seed development as well. Bogue et al. (1990) showed that a 2.4-kb 5′ upstream region of the *Ha*G3-A gene conferred rigorous seed-specific expression to a GUS reporter gene. As expected, the full-length *Ha*G3-A URE GUS fusion, designated *Ha*G3-A-FL, was not expressed in vegetative tissues under any physiological conditions, including exposure to exogenous ABA (Table 3).

The full-length helianthinin *Ha*G3-A URE (−2376 to +24) was tested for its inducibility by ABA in developing seeds. Seeds from transgenic tobacco containing FL-GUS (Figure 1) were staged at 11, 14, 18, and 24 DPF and were tested for their ability to respond to ABA. Induction by ABA was demonstrated by increased levels of GUS activity relative to levels obtained on basal media (see Methods). Results obtained with 14-, 18-, and 24-DPF transgenic tobacco seeds are summarized in Figure 7; seeds from 11 DPF did not respond to ABA during the course of the experiment and, therefore, data are not included. After 12 DPF, ABA responsiveness varied with the stage of development. Seeds from 14 DPF responded rapidly with induction above basal levels beginning as early as 1.5 hr (data not shown; Bogue, 1990). There was a significant increase in GUS activity with 14-DPF seeds treated with ABA; after 3 days of treatment, the levels of GUS activity were higher than those for 18- and 24-DPF seeds with or without ABA. Seeds from 18 DPF were slower to respond to ABA than those from 14 DPF, but levels of GUS activity comparable to 14-DPF (+ABA) seeds were observed in seeds 18 DPF by the fifth day of ABA treatment. Seeds from 24 DPF were less responsive to ABA through 5 days of ABA treatment. Levels of GUS activity also varied with seeds incubated on basal media alone; this increase in the absence of ABA reflects the normal seed protein developmental program.

The preceding results suggested a rigorous regulatory hierarchy controlling helianthinin gene expression, so that the ABA-responsive elements contained within the *Ha*G3-A and *Ha*G3-D UREs are functional only within the context of the appropriate developmental program (i.e., seed maturation). Taking the ABREs out of the context of the *Ha*G3-A or *Ha*G3-D UREs results in the loss of hierarchical control, so that these elements are free to respond directly to ABA and indirectly to desiccation in leaves and seedlings of transgenic tobacco. In the full-length helianthinin URE, these elements only respond to ABA in the developing seed, which suggests that the helianthinin gene contains additional regulatory elements, possibly sequences in the proximal promoter region, that ensure hierarchical control in the developing seed.

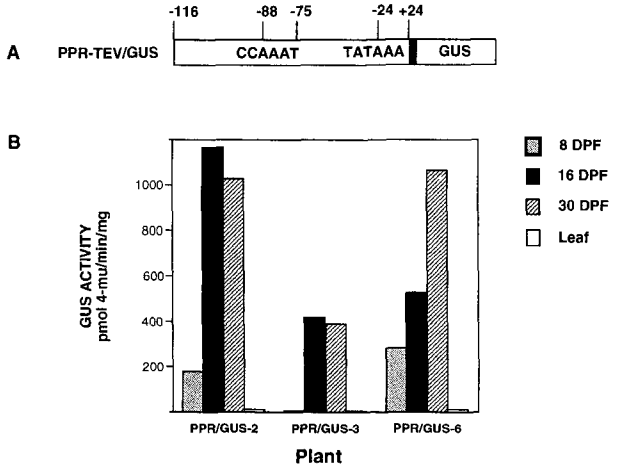

Figure 4. GUS Expression in Transgenic Tobacco Driven by the Helianthinin PPR.

(A) PPR-GUS, including the −116 to +24 region of the helianthinin gene of *Ha*G3-A fused to the chimeric TEV translational enhancer-GUS reporter gene, was transferred to tobacco. The location of the TEV enhancer is indicated by the filled vertical rectangle.

(B) GUS expression in developing seeds and mature leaves of tobacco containing PPR-GUS; 4-MU, 4-methylumbelliferone.

DISCUSSION

A 2.4-kb upstream region of a sunflower gene encoding the seed protein helianthinin confers rigorous developmental GUS expression in transgenic tobacco seeds with no detectable GUS activity in nonembryonic tissues (Bogue et al., 1990). The preceding results implicated two major classes of *cis*-regulatory elements (summarized in Figure 8) in the regulation of the helianthinin genes *Ha*G3-A and *Ha*G3-D; these are seed-specification elements and ABREs. Discrete elements of helianthinin UREs confer ectopic expression patterns when analyzed outside the context of the complete helianthinin regulatory complex, including *cis*-regulatory elements that confer ectopic expression in nonembryonic tissues, and in some cases ABA-responsive gene expression in leaves of mature plants and in leaves and roots of transgenic tobacco seedlings. Although it has been reported that the −90 truncated promoter of CaMV can drive significant GUS expression in the roots of transgenic tobacco (Benfey et al., 1989), we observed no significant expression in transgenic tobacco plants harboring a similar construction. This agrees with our previous study (Jordano et al., 1989) and that of Bustos et al. (1991). Results in Figures 2 to 4 and in Tables 1 and 2 indicate that a PPR is required for seed-specific expression. This region can direct seed-specific expression of the GUS reporter gene (Figure 4); expression is localized primarily to the embryonic apical region and the cotyledons (Figure 3). However, the boundaries of the critical PPRs involved require further definition. Furthermore, based on results with other seed protein genes, more distal

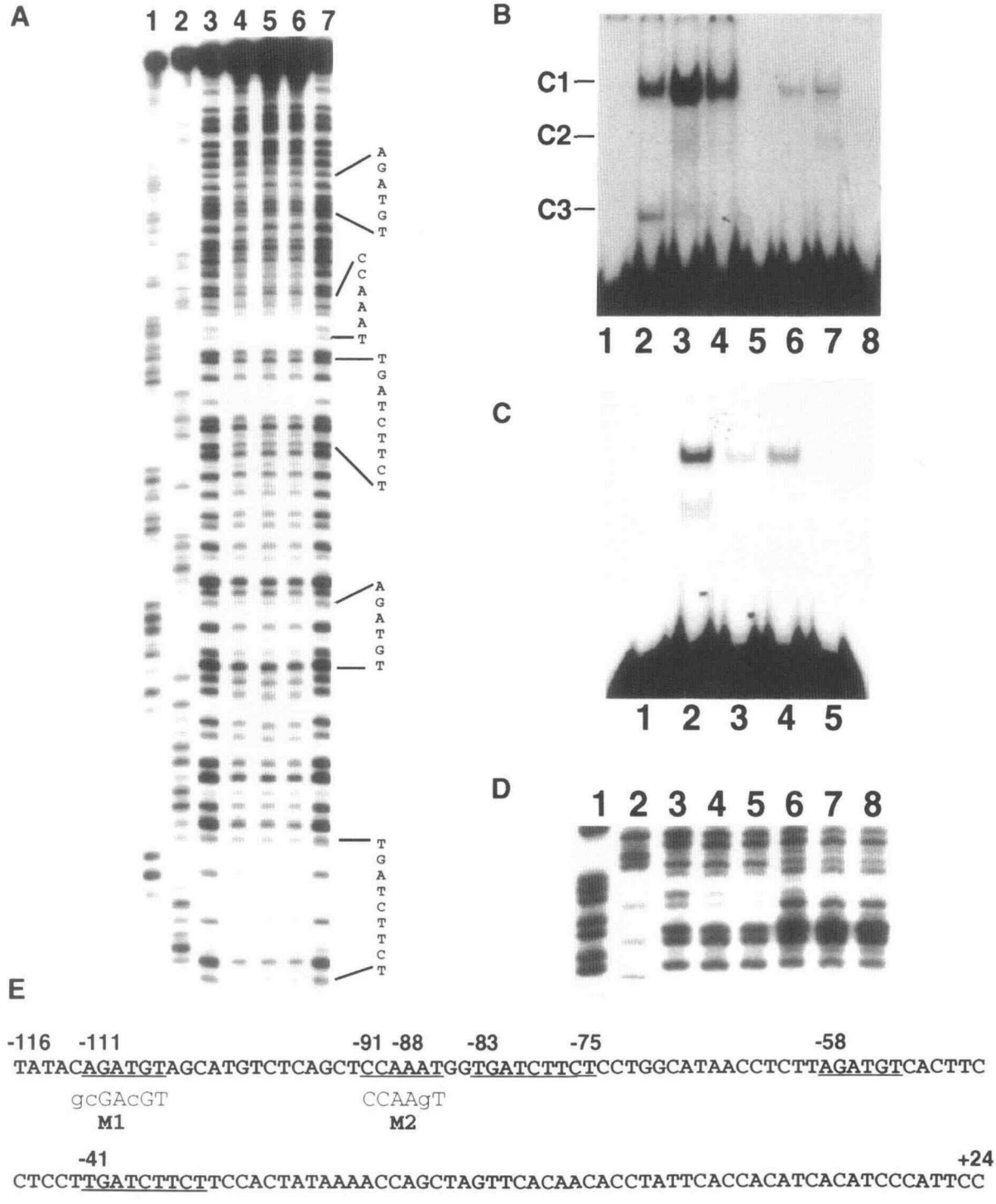

Figure 5. Sunflower Nuclear Proteins Interact with Specific Elements in the Helianthinin PPR.

(A) DNase I protection of the helianthinin PPR. A 5′ end-labeled *Ha*G3-A DNA fragment (−135 to +24) was incubated with 12-DPF nuclear proteins and treated with DNase I. Lanes 1 and 2 contain A+G and T+C Maxam-Gilbert DNA ladders, respectively; lanes 3 and 7, no nuclear proteins added; lanes 4, 5, and 6, 13, 16, and 20 μg of nuclear protein, respectively. Regions protected from DNase I are indicated.

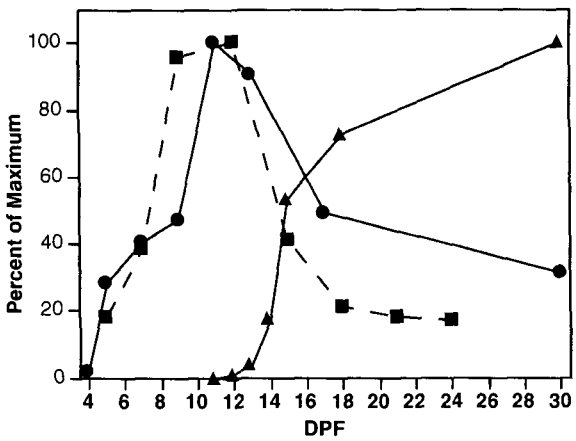

Figure 6. Profiles of GUS Activity and Endogenous ABA Levels in Developing Seeds.

GUS activity and ABA levels in transgenic tobacco containing *Ha*G3-A-FL and *Ha*G3-D-404F were measured. Seeds of transgenic tobacco containing representative forward constructions (see Figure 1) were staged and assayed for GUS activity. Endogenous ABA levels during seed development were also determined. ■, ABA; ▲, FL; ●, 404F.

positive and negative regulatory elements are anticipated (e.g., Bustos et al., 1991).

A minimum of three distinct sequence motifs were identified in the helianthinin PPR that interact with nuclear proteins (Figure 5). Two sequence motifs, AGATGT and TGATCT, each occur twice in the helianthinin PPR: they are AGATGT at −111 and −58 and TGATCT at −83 and −41. Disruption of the helianthinin PPR at −75 of the helianthinin promoter, including the preceding motifs, resulted in loss of GUS expression or ectopic GUS expression in nonembryonic tissues (Table 1). Point mutations in the AGATGT motifs abolished binding to seed nuclear protein(s) and concomitantly reduced the level of PPR-driven GUS expression in transgenic tobacco seeds (Z. Li and T. Thomas, manuscript in preparation). The AGATGT and TGATCT motif, or *WS* motifs, share the consensus sequence WGATST, where W = A or T and designates weak (two) hydrogen bonds, and S = G or C and designates strong (three) hydrogen bonds. We speculate that the *WS* motifs act in concert in the function of the PPR and that an important component of their function is the alteration of the topology of the promoter complex. However, it is also possible that the spacing of these nuclear protein binding sites may be important. The third DNA binding motif identified in the helianthinin PPR, CCAAAT, is similar to the C/EBP binding motif. In animals, C/EBP is involved in communication between upstream enhancers and basal promoter elements (Landschulz et al., 1988), and it is required for embryonic development (Rørth and Montell, 1992).

A combination of sequence identity and functional analysis identified two ABREs in the *Ha*G3-A URE and a single ABRE in the *Ha*G3-D URE (Figure 8). In the full-length helianthinin UREs, these elements only respond to ABA in the developing seed (Figure 7), suggesting that the helianthinin gene contains additional regulatory elements that ensure hierarchical control in the developing seed. Sequences in the ABREs of helianthinin genes share limited similarity to the core consensus sequence CACGTGGC identified in other ABA-responsive genes (Guiltinan et al., 1990; Lam and Chua, 1991). The proposed consensus ABRE in the helianthinin promoters is TACGAACC; it shares less than 75% similarity with the consensus CACGTGGC. So far, proof of function for putative helianthinin ABREs has involved gain-of-function experiments with overlapping 5′ regions from *Ha*G3-A and *Ha*G3-D fused to a truncated CaMV 35S promoter (Table 1; Bogue, 1990). The most definitive identification of specific DNA sequences including a functional ABRE is a 404-bp region (−725 to −322) of the *Ha*G3-D gene. It is noteworthy that the three helianthinin ABREs are immediately adjacent to AT-rich sequences that bind to ubiquitous nuclear proteins, probably HMG chromosomal proteins (Jacobsen et al., 1990; Pedersen et al., 1991; J. Jordano and T. Thomas, unpublished results). A similar AT-rich sequence from the β-phaseolin gene enhances GUS expression in various tissues of transgenic tobacco (Bustos et al., 1989). Thus, it is possible

Figure 5. (continued).

(B) Mutations in the distal AGATGT motif affect binding to sunflower embryo and hypocotyl nuclear proteins. Wild-type and mutant (M1) end-labeled *Ha*G3-A PPR DNA fragments (−116 to −91) in lanes 1 to 4 and lanes 5 to 8, respectively, were incubated with no proteins (lanes 1 and 5), 3 μL of heparin-agarose–fractionated (0.5 M KCl) embryo nuclear proteins (lanes 2 and 6), 3 μL of unfractionated embryo nuclear proteins (lanes 3 and 7), or 3 μL of hypocotyl nuclear proteins (lanes 4 and 8). (Refer to **[E]** for probe identification and location of mutations.)
(C) Distal and proximal AGATGT motifs bind similar nuclear proteins. End-labeled *Ha*G3-A PPR DNA fragments (−116 to −91) were incubated with no protein (lane 1), with 4 μg of embryo nuclear proteins without competitor DNA (lane 2) or in the presence of 50 ng of wild-type competitor (−116 to −91; lane 3), with 50 ng of M1 competitor (−116 to −91; lane 4), or with 150 ng of proximal competitor (−88 to +24; lane 5).
(D) DNase I footprint experiment comparing the interaction of embryo nuclear proteins with the wild-type and mutant CCAAAT motif (M2). A 5′ end-labeled *Ha*G3-A PPR DNA fragment (−116 to +24) was incubated with heparin-agarose–fractionated (0.5 M KCl) embryo nuclear proteins and was then treated with DNase I. Lanes 1 and 2 contain A+G and T+C DNA ladders, respectively; lanes 3 and 6, no nuclear proteins; lanes 4 and 7, 12 μg of nuclear proteins; lanes 5 and 8, 14 μg of proteins. Lanes 3 to 5 contain the wild-type probe; lanes 6 to 8, the M2 probe (refer to **[E]**).
(E) Sequence of helianthinin PPR (−116 to +24). DNase I–protected motifs are underlined; the location of the initial nucleotide is indicated above each motif. Mutated motifs are shown below the wild-type sequence.

Table 3. ABA Induction of Chimeric GUS Reporter Genes in Transgenic Tobacco Seedlings[a]

	GUS Activity (pmol 4-MU/mg/min)					
	*Ha*G3-D-404F			*Ha*G3-A-FL		
ABA Concentration	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
0.1 mM	26 ± 1 (2.4)	94 ± 3 (10)	230 ± 5 (19)	2.8 ± 0.1 (1.1)	2.3 ± 0.1 (1.2)	2.7 ± 0.1 (1.2)
1.0 mM	180 ± 6 (16)	440 ± 10 (48)	860 ± 30 (72)	2.8 ± 0.1 (1.1)	2.2 ± 0.04 (1.1)	2.7 ± 0.1 (1.2)
10.0 mM	1300 ± 10 (120)	2000 ± 20 (220)	2500 ± 60 (210)	2.6 ± 0.1 (1.0)	2.4 ± 0.01 (1.2)	2.4 ± 0.1 (1.0)
Control	11 ± 4	9.1 ± 1	12 ± 1	2.6 ± 0.1	2.0 ± 0.1	2.3 ± 0.1

[a] Transgenic tobacco seedlings (21 DPI) containing the indicated construction were transferred to media containing 0 (control), 0.1, 1.0, or 10 mM ABA. On days 1, 2, and 3 following transfer, samples were removed, and levels of GUS expression were determined. Each value is the average of three determinations. Fold induction relative to the appropriate control is given in parentheses. GUS activity is given in picomoles of 4-methylumbelliferone per milligram per minute.

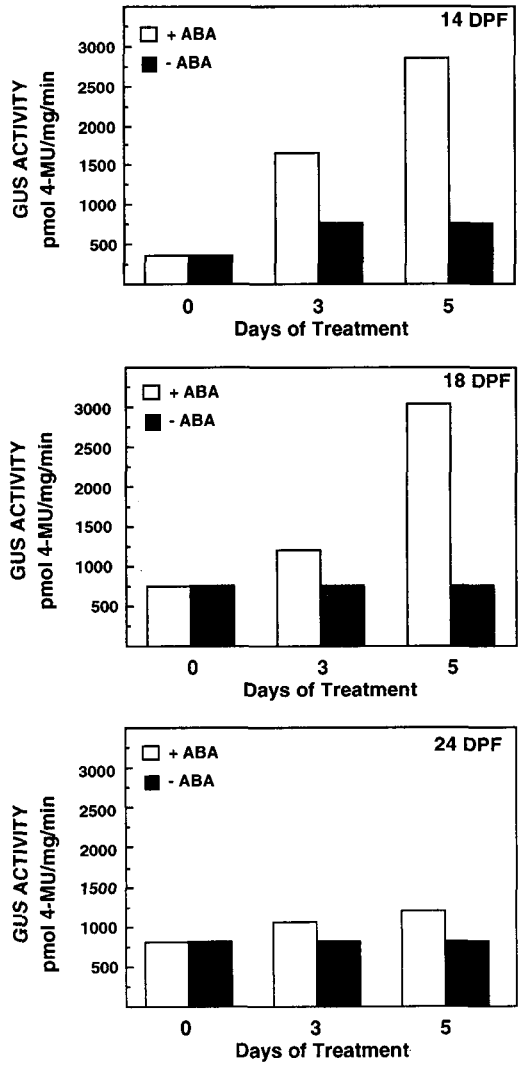

Figure 7. ABA Induction of GUS Expression in Developing Seeds of Transgenic Tobacco Containing *Ha*G3-A-FL.

Seeds were dissected from pods at 11, 14, 18, and 24 DPF and incubated on basal media (−ABA) or basal media containing 1 μM ABA (+ABA). GUS activity was determined 0, 3, and 5 days after treatment. Data are shown for 14, 18, and 24 DPF. 4-MU, 4-methylumbelliferone.

that the ABREs identified in the sunflower helianthinin genes include distantly related congeners of the consensus sequence CACGTGGC and an AT-rich enhancer.

The preceding results as well as results on a carrot late embryo abundant class gene *Dc3* (H. Chung and T. Thomas, unpublished results) suggest a bipartite structure for seed protein gene regulatory ensembles, and possibly for other highly expressed, tissue-specific genes (Figure 8). Proximal promoter elements direct seed- or tissue-specific expression, whereas more distal elements enhance and modulate this basic pattern. PPRs are implicated in seed-specific expression of several seed protein genes (see Goldberg et al., 1989; Bäumlein et al., 1991; Bustos et al., 1991). However, there is limited sequence similarity in the PPRs of the helianthinin genes and other known seed- and embryo-specific genes, and as a consequence, we have been unable to identify significant conserved sequences shared with other seed-specific genes that might facilitate precise identification of seed-specification elements. More distal elements in the helianthinin regulatory ensemble are required to modulate and expand the dynamic range of seed protein gene expression in time and space, including response to fluctuations in ABA concentrations (summarized in Figure 8). These elements, including AT-rich enhancer elements (Jordano et al., 1989) and ABREs, expand the tissue boundaries of helianthinin expression and temporally and quantitatively modulate helianthinin expression. Additional elements maintain hierarchical control over distal ABREs so that in the helianthinin promoters, these elements are only ABA responsive in developing seeds (Thomas et al., 1991; A. Nunberg and T. Thomas, manuscript in preparation).

The UREs of seed protein genes are extensive and complex. Sequences within these regions respond to developmental and hormonal signals, resulting in highly tissue-specific and in

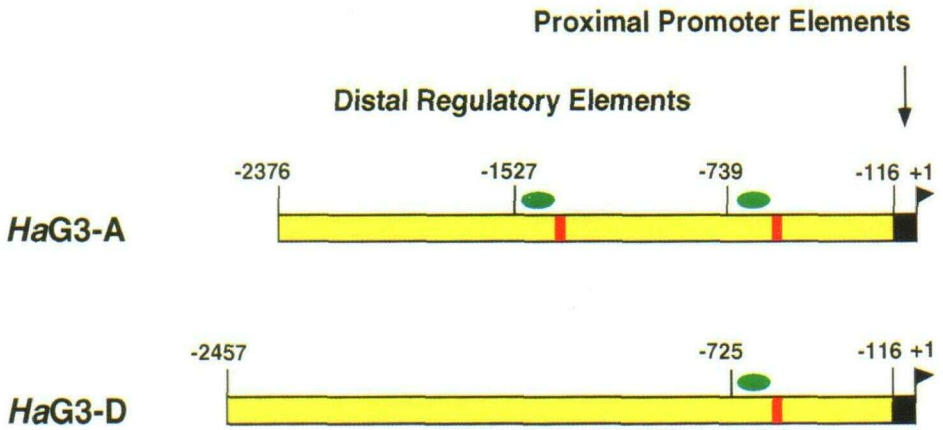

Figure 8. Bipartite Organization of Helianthinin UREs.

UREs of *Ha*G3-A and *Ha*G3-D are shown. PPRs are in black and are indicated by the vertical arrow; distal regulatory regions are in yellow. ABREs are indicated by red vertical rectangles. Green ovals are located above AT-rich motifs (Jordano et al., 1989) that probably bind HMG-like chromosomal proteins.

some cases spatially modulated expression patterns (Goldberg et al., 1989; Perez-Grau and Goldberg, 1989). As is the case with numerous well-studied plant and animal genes, the emerging picture of seed protein regulatory regions is one that includes *cis*-acting regulatory domains that are modular arrangements of shorter DNA segments (Dynan, 1989; Mitchell and Tjian, 1989; Baniahmad et al., 1990; Benfey et al., 1990; Bustos et al., 1991). The expression of a specific gene, in this case a seed protein gene, depends on a combinatorial array of distinct regulatory modules and a specific complement of *trans*-acting factors represented in a given tissue or cell type. Thus, each gene has a unique combination of *cis*-acting DNA sequences that function to direct its expression. In this regard, it is noteworthy that a 22-bp sequence from the PPR of the pea lectin gene confers seed-specific gene expression; the functional element appears to include three overlapping TGAC-like motifs that are binding sites for basic–leucine zipper proteins (de Pater et al., 1993). The helianthinin PPR, which also confers seed-specific expression, does not contain these motifs, but rather has a complement of different *cis*-acting DNA sequences that interact with a different class of nuclear factors, probably zinc finger proteins, to confer tissue-specific expression (Z. Li and T. Thomas, unpublished results). These results appear to confirm the combinatorial nature of seed-specific promoters, and although the combinatorial code required to assemble *cis*-regulatory modules into transcriptional language is currently not apparent, experimental systems are now in hand to solve this problem in detail.

METHODS

β-Glucuronidase Reporter Gene Constructions

β-Glucuronidase (GUS) reporter cassettes used throughout were in pBIN19 (Bevan, 1984; Jefferson et al., 1987). The fragments containing helianthinin *Ha*G3-A and *Ha*G3-D upstream regions used for GUS constructions are shown in Figure 1. The *Ha*G3-A-GUS constructions represent large overlapping fragments that span the full-length (FL) regulatory region (−2376 to +24), which was described previously (Bogue et al., 1990). The 3′ ends of several constructions were derived from exonuclease III digestions of a 2.8-kb *Ha*G3-A fragment in pBluescript SK+ (Stratagene) (Bogue et al., 1990). These deletions are shown at the top of Figure 1. The first deletion, p*Ha*G3-A-2.4 (BamHI-Δ1), contains the *Ha*G3-A CAAT and TATA boxes with its 3′ end at +24; a second deletion, p*Ha*G3-A-2.3 (Bam HI-Δ2), contains only the *Ha*G3-A CAAT box with its 3′ end at −75. Fragments that contained the *Ha*G3-A CAAT and TATA boxes were ligated in forward orientation into the promoterless GUS cassette pBI101.1. Fragments that did not contain the *Ha*G3-A TATA box were ligated in both orientations upstream of the truncated cauliflower mosaic virus (CaMV) 35S promoter of pBI120, resulting in a chimeric promoter–enhancer complex driving the expression of GUS. Constructions are named according to their terminal restriction sites followed by an F, indicating forward orientation, or R, indicating reverse orientation. Arrows indicate the orientation of the fragment with respect to the GUS coding region (Figure 1).

The *Ha*G3-D-404 constructions contain a 404-bp fragment (SalI-HpaI) inserted in both orientations in pBI120 (Jordano et al., 1989). Proximal promoter region (PPR)–GUS was constructed as follows. The PPR (−116 to +24) from *Ha*G3-A (Bogue et al., 1990) was synthesized using mutually priming oligonucleotides with endonuclease restriction sites at the 5′ ends and Klenow fragment of DNA polymerase I (Ausubel et al., 1987). The resulting synthetic DNA fragment was digested with BamHI and EcoRI and subsequently cloned in pBluescript SK+ to create p*Ha*G3-A-116. An EcoRI–HindIII fragment containing a tobacco etch virus (TEV) translational enhancer and nopaline synthase (*nos*) terminator from pRTL2.4G (Carrington and Freed, 1990) was inserted 3′ of PPR to generate PPR–GUS in pBluescript SK+. A SacI-HindIII PPR–GUS fusion fragment was then cloned into pBin19. 5′ deletions −1527, −739, −651, and −321 were generated by restriction digests of the plasmid p*Ha*G3-2.4 (BamHI-Δ1) and then subsequently cloned in the forward orientation into the promoterless GUS cassette pBI201.1, a pUC19 derivative of pBI101.1. The GUS fusion was then cloned into pBin19. The −116 deletion was made by subcloning the insert of p*Ha*G3-A-116 into pBI201.1. The 116–GUS fusion was then moved into pBin19.

The −74 deletion was made by 5′ exonuclease III digestion of the full-length *Ha*G3-A-GUS fusion. The orientation of each construction was confirmed by double-stranded dideoxy sequencing (Chen and Seeburg, 1985) using primers to regions in the GUS cassettes (Gene Technologies Laboratory, Texas A&M University); sequence analysis was done with the GCG DNA sequence analysis software (Devereux et al., 1984) on a DEC MicroVAXII.

Plant Material

pBin19-based plasmid constructions were used to transform tobacco (*Nicotiana tabacum* cv Xanthi) according to standard procedures (Horsch et al., 1985; Bogue et al., 1990), except that initial transformants were selected on 50 μg/mL kanamycin and then were transferred to 100 μg/mL kanamycin. Plants were self-pollinated, and seeds were germinated on kanamycin (400 μg/mL). The copy number of each GUS construction integrated into the tobacco genome was estimated for each transformant by segregation frequencies of the neomycin phosphotransferase II gene and confirmed by genomic DNA gel blots. Most of the transformants contained only one segregating locus of each construction. Transgenic plants were maintained in Conviron chambers: 16-hr light/8-hr dark at 24°C and 70 to 80% relative humidity. All plants were watered on a strict schedule to prevent desiccation.

Transgenic tobacco seeds were staged according to days postflowering (DPF). Seeds less than 24 DPF were taken directly from the plant and manipulated under sterile conditions. Desiccated seeds from 24 and 35 DPF were surface sterilized and imbibed in water for 1 hr prior to treatment. Seeds from each stage were taken from the same seed pod to ensure synchronous development and were placed on basal media ([MS salts; Murashige and Skoog, 1962], 0.8% inositol, 10 μg/mL thiamine-HCl, 0.2% Gel-Rite [Scott Laboratories, Inc., Carson, CA]) with or without abscisic acid (ABA) (+/− *cis/trans* isomer; Sigma). For determinations of endogenous ABA in developing seeds, staged pods of transgenic tobacco were collected and immediately frozen in liquid nitrogen and lyophilized; ABA extraction and determination was by gas liquid chromatography as described by Creelman et al. (1990).

Biochemical and Histochemical Detection of GUS Activity

The standard procedures of Jefferson et al. (1987) were followed (see Bogue et al., 1990 for details). Biochemical assays were performed by mixing plant tissue lysates with an equal volume of 2 mM 4-methylumbelliferyl β-D-glucuronide and incubating for 1 hr at 37°C. Fluorometric analyses were done with a minifluorometer (model TKO-100; Hoefer Scientific Instruments, San Francisco, CA) as described previously (Jefferson, 1987). Protein concentrations were determined by the method of Bradford (1976). Histochemical localizations for GUS activity were determined by incubating whole tissue in 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as described by Jefferson (1987). Potassium ferricyanide and potassium ferrocyanide were added as previously described (Bogue et al., 1990). X-gluc treatment was carried out for 18 hr at 37°C. Samples were mounted on microscope slides with 80% glycerol and photographed with Kodak Ektachrome 160 ASA tungsten film.

Mutagenesis

The mutant M2 (CCAAgT) was created by random polymerase chain reaction (PCR) mutagenesis using p*Ha*G3-A-116 as template and SK and KS primers (Stratagene); the deoxynucleotide triphosphate concentration was at 80 μM except for dCTP, which was at 8 μM. The mutant p*Ha*G3-A-116M2 was identified by double-stranded DNA sequencing. The M1 mutant was created by PCR using the synthesized mutant oligonucleotide 5′-TCGGATCCTATAgcGAcGTAGCAT-3′ spanning −116 to −92. The PCR product was cloned into pBluescript SK+ (Stratagene) to generate p*Ha*G3-A-116M1. To generate the −116 to −91 probe and −88 to +24 competitor, two PCR-linker scanning mutants (Gustin and Burk, 1993) were made using two oligonucleotides (5′-CAGCtctagaTGGTGATC-3′ and 5′-ACCAtctagaGCTGAGAC-3′) and p*Ha*G3-A-116 and p*Ha*G3-A-116M1 as templates; these were designated p*Ha*G3-A-116Xba and p*Ha*G3-A-M1Xba. The resulting mutants created an XbaI site at −92.

Analysis of DNA Protein Interactions

Nuclear extracts were prepared from sunflower embryos 12 DPF or hypocotyl according to the methods described by Jordano et al. (1989). The sunflower embryo nuclear extracts were precipitated by $(NH_4)_2SO_4$, pH 7.9, at 20,000*g* for 20 min at 4°C. The fraction from 20 to 60% saturation was dissolved in 0.05 M KCl, TM buffer (TM buffer is 50 mM Tris-HCl, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20% glycerol), and applied to a heparin–agarose column (Briggs et al., 1986). Bound protein was eluted sequentially with TM buffer containing 0.1 M, 0.5 M, and 1.0 M KCl; 0.5-M KCl fractions were pooled and used for further experiments. End-labeled DNA fragments (~0.1 ng, 10,000 cpm) were incubated with nuclear proteins and 1.0 μg of poly(dI-dC) poly(dI-dC) (Pharmacia). Binding reactions were performed for 15 min at 25°C in 10 μL of 15 mM Hepes, pH 7.9, 15% glycerol, 25 mM KCl, 15 mM $MgCl_2$, 0.25 mM phenylmethylsulfonyl fluoride, and 1.2 mM DTT. For competition experiments, binding reactions also included unlabeled DNA fragments. After binding, reaction mixtures were separated by electrophoresis in 8% polyacrylamide gels in 33% Tris-borate-EDTA, pH 7.9, at room temperature.

For DNase I footprinting experiments, binding reactions were scaled up to a final volume of 30 μL with 20,000 cpm asymmetrically end-labeled DNA fragment. After binding for 15 min at room temperature, 5 μL of DNase I was added (0.004 to 0.01 units; Promega) and incubated at room temperature for 1.5 min. The reaction was terminated by adding 80 μL of DNase I stop solution (450 mM NaOAc, 0.15 μg tRNA, 5 mM EDTA, 0.5 μg proteinase K, 1.6% SDS), incubated at 37°C for 45 min, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol. DNA was analyzed on 10% polyacrylamide sequencing gels with Maxam and Gilbert (1980) sequencing ladders prepared from the same end-labeled fragments.

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1987). Current Protocols in Molecular Biology. (New York: Wiley Interscience).

Baniahmad, A., Steiner, C., Köhne, A.C., and Renkawitz, R. (1990). Modular structure of a chicken lysozyme silencer: Involvement of an unusual thyroid hormone receptor binding site. Cell **61,** 505–514.

Bäumlein, H., Boerjan, W., Nagy, I., Panitz, R., Inzé, D., and Wobus, U. (1991). Upstream sequences regulating legumin gene expression in heterologous transgenic plants. Mol. Gen. Genet. **225,** 121–128.

Benfey, P.N., Ren, L., and Chua, N.-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. **8,** 2195–2202.

Benfey, P.N., Ren, L., and Chua, N.-H. (1990). Combinatorial and synergistic properties of CaMV 35S enhancer subdomains. EMBO J. **9,** 1685–1696.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res. **12,** 8711–8721.

Bogue, M.A. (1990). Analysis of *cis*-Regulatory Elements Controlling the Expression of Two Sunflower 11S Globulin Genes. Ph.D. Dissertation (College Station: Texas A & M University).

Bogue, M.A., Vonder Haar, R.A., Nuccio, M.L., Griffing, L.R., and Thomas, T.L. (1990). Developmentally regulated expression of a sunflower 11S seed protein gene in transgenic tobacco. Mol. Gen. Genet. **221,** 49–57.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Bray, E.A., and Beachy, R.N. (1985). Regulation by ABA of β-conglycinin expression in cultured developing soybean cotyledons. Plant Physiol. **79,** 746–750.

Briggs, M.R., Kadonaga, J.T., Bell, S.P., and Tjian, R. (1986). Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. Science **234,** 47–52.

Bustos, M.M., Guiltinan, M.J., Jordano, J., Begum, D., Kalkan, F.A., and Hall, T.C. (1989). Regulation of β-glucuronidase expression in transgenic tobacco plants by an A/T-rich, *cis*-acting sequence found upstream of a French bean β-phaseolin gene. Plant Cell **1,** 839–853.

Bustos, M., Begum, D., Kalkan, F.A., Battraw, M.J., and Hall, T.C. (1991). Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. EMBO J. **10,** 1469–1479.

Carrington, J.C., and Freed, D.D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. **64,** 1590–1597.

Carrington, J.C., Freed, D.D., and Leinicke, A.J. (1991). Bipartite signal sequence mediates nuclear translocation of the plant potyviral NIa protein. Plant Cell **3,** 953–962.

Chen, E.Y., and Seeburg, P.H. (1985). Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. DNA **4,** 165–170.

Chen, Z.L., Pan, N.-S., and Beach, R.N. (1988). A DNA sequence element that confers seed-specific enhancement to a constitutive promoter. EMBO J. **7,** 297–302.

Creelman, R.A., Mason, H.S., Bensen, R.J., Boyer, J.S., and Mullet, J.E. (1990). Water deficit and abscisic acid cause differential inhibition of shoot versus root growth in soybean seedlings. Analysis of growth, sugar accumulation, and gene expression. Plant Physiol. **92,** 205–214.

de Pater, S., Pham, K., Chua, N.-H., Memelink, J., and Kijne, J. (1993). A 22-bp fragment of the pea lectin promoter containing essential TGAC-like motifs confers seed-specific gene expression. Plant Cell **5,** 877–886.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequences and analysis programs for the VAX. Nucl. Acids Res. **12,** 287–395.

Dynan, W.S. (1989). Modularity in promoters and enhancers. Cell **58,** 1–4.

Finkelstein, R.R., Tenbarge, K.M., Shumway, J.E., and Crouch, M.L. (1985). Role of ABA in maturation of rapeseed embryos. Plant Physiol. **78,** 630–636.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell **56,** 149–160.

Guiltinan, M.J., Marcotte, W.R., and Quatrano, R.S. (1990). A leucine zipper protein that recognizes an abscisic acid response element. Science **250,** 267–270.

Gustin, K.E., and Burk, R.D. (1993). A rapid method for generating linker scanning mutants utilizing PCR. Biotechniques **14,** 22–23.

Horsch, R., Fry, J., Hoffman, N., Eichholtz, D., Rogers, S., and Fraley, R. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Jacobsen, K., Laursen, N.B., Jensen, E.Ø., Marcker, A., Poulsen, C., and Marcker, K.A. (1990). HMG I-like proteins from leaf and nodule nuclei interact with different AT motifs in soybean nodulin promoters. Plant Cell **2,** 85–94.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS reporter gene fusion system. Plant Mol. Biol. Reporter **5,** 387–405.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene marker in higher plants. EMBO J. **6,** 3901–3907.

Jordano, J., Almoguera, C., and Thomas, T.L. (1989). A sunflower helianthinin gene upstream sequence ensemble contains an enhancer and sites of nuclear protein interaction. Plant Cell **1,** 855–866.

Lam, E., and Chua, N.-H. (1991). Tetramer of a 21-base pair synthetic element confers seed expression and transcriptional enhancement in response to water stress and abscisic acid. J. Biol. Chem. **266,** 17131–17135.

Landschulz, W.H., Johnson, P.F., and Mcknight, S.L. (1988). Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2,** 786–800.

Maxam, A.M., and Gilbert, W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65,** 499–560.

Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245,** 371–378.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant **15,** 473–497.

Pedersen, T.J., Arwood, L.J., Spiker, S., Guiltinan, M.J., and Thompson, W.F. (1991). High mobility group chromosomal proteins bind to AT-rich tracts flanking plant genes. Plant Mol. Biol. **16,** 95–104.

Peña-Cortés, H., Sánchez-Serrano, J.J., Mertens, R., Willmitzer, L., and Prat, S. (1989). Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc. Natl. Acad. Sci. USA **86,** 9851–9855.

Perez-Grau, L., and Goldberg, R.B. (1989). Soybean seed protein genes are regulated spatially during embryogenesis. Plant Cell **1,** 1095–1109.

Quatrano, R.S. (1987). The role of hormones during seed development. In Plant Hormones and Their Role in Plant Growth and Development, P.J. Davies, ed (Boston: Martinus Nijhoff Publishers), pp. 494–514.

Rivin, C.J., and Grudt, T. (1991). Abscisic acid and the developmental regulation of embryo storage proteins in maize. Plant Physiol. **95,** 358–365.

Rórth, P., and Montell, D.J. (1992). Drosophila C/EBP: A tissue specific DNA binding protein required for embryonic development. Genes Dev. **6,** 2299–2311.

Shotwell, M.A., and Larkins, B.A (1989). The biology of seed storage proteins. In The Biochemistry of Plants, A. Marcus, ed (New York: Academic Press), pp. 297–345.

Skriver, K., and Mundy, J. (1990). Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2,** 503–512.

Thomas, T.L. (1993). Gene expression during plant embryogenesis and germination: An overview. Plant Cell **5,** 1401–1410.

Thomas, T.L., Vivekananda, J., and Bogue, M.A. (1991). ABA regulation of gene expression in embryos and mature plants. In Abscisic Acid: Physiology and Biochemistry, W.J. Davies and H.G. Jones, eds (Oxford: Bios Scientific Publishers), pp. 125–135.