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 HaG3-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).
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 HaG3-A and HaG3-D. A 2.4-kb upstream region of the helianthinin gene HaG3-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 transgenic tobacco, expression was driven by sequences derived from the UREs of helianthinin genes HaG3-A and HaG3-D.

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 HaG3-A conferred rigorous, seed-specific expression of the GUS reporter gene (Bogue et al., 1990). The upstream regions of sunflower helianthinin genes HaG3-A and HaG3-D contain extensive regions of similarity (Bogue et al., 1990), as illustrated in Figure 1. For example, the HaG3-D helianthinin gene contains a 404-bp region that is 98% similar to a sequence present in the HaG3-A upstream region with the 5′ end at the Sall 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 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). HaG3-A-FL and HaG3-A-S-Δ1 are transcriptional GUS fusions beginning with +24 of the HaG3-A URE and the promoterless GUS reporter gene pBI101.1 (Jefferson et al., 1997). All other helianthinin GUS reporter genes contain chimeric promoters derived from the HaG3-A or HaG3-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 HaG3-A and HaG3-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’ ) locus. Constructions including the helianthinin proximal promoter elements and a minimum of 1.5 kb of the HaG3-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

<table>
<thead>
<tr>
<th>Construct</th>
<th>Relative GUS Activity (%)</th>
<th>ABA Responsive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Seed</td>
<td>Leaf</td>
</tr>
<tr>
<td>HaG3-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL (-2376 to +24)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>S-Δ1 (-1527 to +24)</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>S-Δ2 (-1527 to -75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>190</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>111</td>
<td>2.6</td>
</tr>
<tr>
<td>B-Δ2 (-739 to -75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>58</td>
<td>11</td>
</tr>
<tr>
<td>R</td>
<td>38</td>
<td>22</td>
</tr>
<tr>
<td>SB (-1527 to -739)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>63</td>
<td>1.6</td>
</tr>
<tr>
<td>HaG3-D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>404 (-725 to -322)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>48</td>
<td>0.5</td>
</tr>
<tr>
<td>R</td>
<td>48</td>
<td>11</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBI101</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pBI120</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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 pBI1120 (refer to text and Figure 1).

Helianthinin Proximal Promoter Region Confers Regionalized GUS Expression in Seeds

The proximal promoter regions (PPRs) of helianthinin genes HaG3-A and HaG3-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 2B, 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 R0 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 HaG3-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 (−116 to −91) 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 (gGAcGCT) 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 (CAGGfT) 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.

**Elements of Helianthinin Promoter Regions Respond to ABA**

Analysis of GUS fusions containing discrete elements of the HaG3-A and HaG3-D UREs identified two regions containing ABA-responsive cis-regulatory elements (ABREs) in the URE of HaG3-A and one ABRE-containing region in HaG3-D (Table 1). Jordano et al. (1989) cloned a region containing the only functionally identified ABRE from HaG3-D (Figure 1) into a GUS cassette and transferred it into tobacco. We found that this construct, called HaG3-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 HaG3-D-404 and HaG3-A-FL in developing tobacco seeds and found that HaG3-D-404 exhibited ectopic expression in developing tobacco seeds when compared to expression of HaG3-A-FL (Figure 6). The results represented two distinct developmental profiles based on the time of initial appearance of GUS activity in developing

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**Table 2. The Helianthinin PPR Directs Seed-Specific GUS Expression**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Leaf</th>
<th>8 DPF</th>
<th>16 DPF</th>
<th>30 DPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>1.2 ± 0.95</td>
<td>3.4 ± 2.8</td>
<td>514 ± 120</td>
<td>415 ± 220</td>
</tr>
<tr>
<td>−739</td>
<td>2.3 ± 0.74</td>
<td>14.4 ± 5.8</td>
<td>797 ± 300</td>
<td>527 ± 138</td>
</tr>
<tr>
<td>−116</td>
<td>1.3 ± 1.0</td>
<td>7.0 ± 0.95</td>
<td>27 ± 11</td>
<td>21 ± 9.6</td>
</tr>
<tr>
<td>−74</td>
<td>1.8 ± 0.52</td>
<td>54.0 ± 8.3</td>
<td>38 ± 11</td>
<td>23 ± 11</td>
</tr>
</tbody>
</table>

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.
embryos and the qualitative and quantitative characteristics of the resulting expression patterns. As shown previously (Bogue et al., 1990), the full-length helianthinin URE (HaG3-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 HaG3-D-404 driven GUS expression occurs by 4 DPF and reaches a peak at \( \sim 10 \) to 12 DPF followed by a rapid decline to less than one-third of the maximal level at 30 DPF. Expression of HaG3-D-404-GUS correlated with elevated levels of ABA during seed development preceding the maturation and desiccation phase of seed development. As expected, HaG3-A-FL expression appears to reflect these later programs.

Results in Table 1 and Figure 6 indicated that the \(-725\) to \(-322\) region of HaG3-D contained an ABRE. The ABRE within HaG3-D-404 responded to endogenous and exogenous ABA in other vegetative tissues, including seedlings. Transgenic tobacco seedlings (21 days postimbition [DPI]) containing HaG3-D-404 and HaG3-A-FL were transferred to media containing 0 to 10 mM ABA, and GUS activity was subsequently determined (Table 3). Seedlings containing HaG3-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 HaG3-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 HaG3-A gene conferred rigorous seed-specific expression to a GUS reporter gene. As expected, the full-length HaG3-A URE GUS fusion, designated HaG3-A-FL, was not expressed in vegetative tissues under any physiological conditions, including exposure to exogenous ABA (Table 3).

The full-length helianthinin HaG3-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 HaG3-A and HaG3-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 HaG3-A or HaG3-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.

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 HaG3-A and HaG3-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 HaG3-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.
Developing Seeds.

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

GUS activity and ABA levels in transgenic tobacco containing HaG3-A-FL and HaG3-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 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 (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 HaG3-A URE and a single ABRE in the HaG3-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 CACGTG-GC 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 CACGTG-GGC. So far, proof of function for putative helianthinin ABREs has involved gain-of-function experiments with overlapping 5' regions from HaG3-A and HaG3-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 HaG3-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 HaG3-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 KCI) embryo nuclear proteins (lanes 2 and 6), 3 µL of unfractinated 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 HaG3-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 HaG3-A PPR DNA fragment (–116 to +24) was incubated with heparin-agarose-fractionated (0.5 M KCI) 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 nuclear 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

<table>
<thead>
<tr>
<th>ABA Concentration</th>
<th>HaG3-D-404F</th>
<th>HaG3-A-FL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>26 ± 1</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>180 ± 6</td>
<td>440 ± 10</td>
</tr>
<tr>
<td>10.0 mM</td>
<td>1300 ± 10</td>
<td>2000 ± 20</td>
</tr>
<tr>
<td>Control</td>
<td>11 ± 4</td>
<td>9.1 ± 1</td>
</tr>
</tbody>
</table>

* 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.

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 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 6). 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; Baumlein 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
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 (Dyman, 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 HaG3-A and HaG3-D upstream regions used for GUS constructions are shown in Figure 1. The HaG3-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 HaG3-A fragment in pBluescript SK+ (Stratagene) (Bogue et al., 1990). These deletions are shown at the top of Figure 1. The first deletion, pHaG3-A-2.4 (BamHI-A1), contains the HaG3-A CAAT and TATA boxes with its 3' end at +24; a second deletion, pHaG3-A-2.3 (BamHI-A2), contains only the HaG3-A CAAT box with its 3' end at -75. Fragments that contained the HaG3-A CAAT and TATA boxes were ligated in forward orientation into the promoterless GUS cassette pBI101.1. Fragments that did not contain the HaG3-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 HaG3-D-404 constructions contain a 404-bp fragment (Sall-Hpall) 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 HaG3-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 pHaG3-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 pHaG3-2.4 (BamHI-A1) 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 pHaG3-A-116 into pBI201.1. The 116–GUS fusion was then moved into pBIN19.
The mutant M2 (CCAAgT) was created by random polymerase chain reaction (PCR) mutagenesis using pHtG3-A-116M2 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 pHtG3-A-116M2 was identified by double-stranded DNA sequencing. The M1 mutant was created by PCR using the synthesized mutant oligonucleotide 5’TCGGATCTCTAGGTGATGACAT-3’ spanning −116 to −92. The PCR product was cloned into pBluescript SK+ (Stratagene) to generate pHtG3-A-116M1. To generate the −116 to −91 probe and −88 to −82 competitor, two PCR-linker scanning mutants (Gustin and Burk, 1993) were made using two oligonucleotides (5’-CAGCtagt8GTTGATC-3’ and 5’-ACCAGtagtGCTAGAC-3’ and pHtG3-A-116 and pHtG3-A-116M1 as templates; these were designated pHtG3-A-116Xba and pHtG3-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 (NH₄)₂SO₄, pH 7.9, at 20,000g for 20 min at 4°C. DNA binding was done in 0.05 M KCl. The fraction from 20 to 60% saturation was dissolved in 0.05 M Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 2.5% p-mercurymercury (pH 7.9, at 20,000g for 20 min at 4°C). The fraction from 20 to 60% saturation was dissolved in 0.05 M Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 2.5% p-mercurymercury, 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 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 10 μg of polyclonal protein (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 MgCl₂, 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 Tnase, 5 mM EDTA, 0.5 μg proteinase K, 1% SDS), incubated at 37°C for 45 min, extracted with phenol-chloroform-isomyl 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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Regulation of Sunflower Helianthinin Genes


Developmental and hormonal regulation of sunflower helianthinin genes: proximal promoter sequences confer regionalized seed expression.

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