Homeodomain Leucine Zipper Proteins Bind to the Phosphate Response Domain of the Soybean VspB Tripartite Promoter

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The soybean (Glycine max L. Merr. cv Williams 82) genes VspA and VspB encode vacuolar glycoprotein acid phosphatases that serve as vegetative storage proteins during seed fill and early stages of seedling growth. VspB expression is activated by jasmonates (JAs) and sugars and down-regulated by phosphate and auxin. Previous promoter studies demonstrated that VspB promoter sequences between −585 and −535 mediated responses to JA, and sequences between −535 and −401 mediated responses to sugars, phosphate, and auxin. In this study, the response domains were further delineated using transient expression of VspB promoter-β-glucuronidase constructs in tobacco protoplasts. Sequences between −536 and −484 were identified as important for phosphate responses, whereas the region from −486 to −427 mediated sugar responses. Gel-shift and deoxyribonuclease-I footprinting assays revealed four DNA-binding sites between −611 and −451 of the soybean VspB promoter: one in the JA response domain, two in the phosphate response domain, and one binding site in the sugar response domain. The sequence CATTAAATTAG present in the phosphate response domain binds soybean homeodomain leucine zipper proteins, suggesting a role for these transcription factors in phosphate-modulated gene expression.

The soybean (Glycine max L. Merr. cv Williams 82) vegetative storage proteins VSP\textsuperscript{a} and VSP\textsuperscript{b} are vacuolar acid phosphatases that accumulate in hypocotyl hooks and plumes during seedling development, and in leaves, stems, and pods during the reproductive phase (Wittenbach, 1982; Staswick, 1989a; DeWald et al., 1992). Sink deprivation by dedoping or petiole girdling also causes a massive accumulation of the Vsp mRNAs and proteins (Wittenbach, 1982; Staswick, 1989b). The accumulation of the VSP in vacuoles of cells in sink tissues and in response to dedopping led Wittenbach (1983) to identify these proteins as vegetative storage proteins.

Vsp mRNA levels are highest in the plumule, hypocotyl hook, hypocotyl elongation region, and young leaves, whereas low levels are detected in more mature portions of the hypocotyl, older leaves, and roots (Mason and Mullet, 1990). Vsp expression is also high in buds and flowers of Arabidopsis (Berger et al., 1995). Tissues with high levels of Vsp mRNA have elevated levels of jasmonates (JAs; Mason et al., 1992; Creelman and Mullet, 1995), linolenic acid-derived compounds involved in plant defense (Vick and Zimmerman, 1984; Gundlach et al., 1992; Hamberg and Gardner, 1992; Sembdner and Parthier, 1993). Treatment of leaves or soybean cell cultures with JA causes accumulation of Vsp mRNA and protein (Anderson, 1988; Anderson et al., 1989; Mason and Mullet, 1990). Vsp expression and JA levels increase in response to wounding (Creelman et al., 1992; Albrecht et al., 1993), and Vsp expression is absent in mutants that cannot respond (Feyes et al., 1994) or synthesize JA (McConn et al., 1997).

Vsp expression is stimulated by sugars and repressed by phosphate and auxin (DeWald et al., 1994). Full induction of Vsp mRNA accumulation in excised mature soybean leaves required 10 μM methyl jasmonate (MeJA) plus illumination or 0.2 mM Suc (Mason et al., 1992). In soybean cell suspension culture, Suc, Fru, or Glc plus 10 μM MeJA were needed to achieve maximum induction of VspB (Mason et al., 1992). These experiments showed that Vsp expression is synergistically activated by a combination of JA and sugars. In addition, accumulation of VspB mRNA in soybean cell cultures was inhibited when the phosphate concentration of the growth medium was increased from 0.31 mM to 2.5 mM (Sadka et al., 1994). Plants fed with Man to reduce cytoplasmic phosphate levels also showed increased expression of VspB (Sadka et al., 1994).

The VspB promoter has been characterized by promoter deletion analysis in transgenic tobacco (Nicotiana tabacum cv Samsun; Mason et al., 1993) and by analysis of promoter domains in protoplasts (Sadka et al., 1994).
et al., 1994). The VspB promoter domain from –787 to –289 responded to JA, sugars, and phosphate. Deletion of the promoter to –520 eliminated JA-modulated transcription. Gain-of-function experiments identified a 50-bp DNA sequence, from –585 to –535 of the VspB promoter, that could mediate JA responses when fused to the truncated (–88) 35S-cauliflower mosaic virus (CaMV) promoter (Mason et al., 1993). Other studies showed that the promoter region from –536 to –403 mediated responses to phosphate (inhibition), sugars (stimulation), and auxin (inhibition; DeWald et al., 1994; Sadka et al., 1994). Therefore, this approximately 185-bp portion of the VspB promoter provides an opportunity to identify cis- and trans-factors mediating each response and to study how various sub-domains of this promoter interact to regulate VspB transcription.

In this study, we further define the VspB promoter domains, which mediate responses to phosphate and sugar using transient expression assays in tobacco protoplasts. Gel shift and deoxyribonuclease (DNase)-I footprinting assays were used to map protein-binding sites in each of the promoter domains to obtain a more detailed understanding of the architecture of this promoter. Site-directed mutagenesis of a protein-binding site in the phosphate response domain confirmed the importance of the sequence CATTAATTAG in trans-factor binding. Two soybean genes were identified encoding homeodomain (HD)-Leu zipper proteins (ZIPs) that bind to this sequence.

RESULTS
Sugar and Phosphate Response Domains in the Soybean VspB Promoter

The VspB promoter region from –585 to –535 was previously shown to mediate responses to JA in transgenic plants (Mason et al., 1993), and the region from –536 to –401 was shown to mediate responses to sugars and phosphate in protoplasts (Sadka et al., 1994). To further analyze the architecture of the VspB promoter, several additional constructs, shown in Figure 1, were made by inserting PCR-amplified DNA fragments of the VspB promoter in front of a truncated (–88) 35S-CaMV promoter of pBI232. These constructs were introduced into tobacco leaf protoplasts by electroporation and after various treatments, GUS activity was assayed.

Protoplast assays were selected for this analysis because experimental results from each assay represent an average response from many protoplasts. In contrast, results from transgenic plants vary depending on site of insertion. We note, however, that although the general responses observed with different constructs were reproducible, the magnitude of the responses varied in different protoplast preparations for unknown reasons. This variation was observed even though we used a second construct containing the CAT reporter gene driven by the 35S-CaMV promoter as an internal standard (Fromm et al., 1986), and expressed results as the relative ratio of GUS/CAT activity for each treatment. Therefore, proto-

![Figure 1. VspB promoter constructs and transient expression assays.](image-url)

The expression vector pBI232 was constructed by replacing the 800-bp CaMV 35S-promoter of pBI221 (CLONTECH Laboratories, Palo Alto, CA) with a minimal (–88) 35S-CaMV promoter followed by the tobacco etch virus 5’-untranslated leader sequence. Various regions of the VspB promoter were inserted upstream of the minimal (–88) 35S-promoter in pBI232 to make the constructs used in transient expression assays (shown at the left). Protoplasts were cotransfected with a CaMV promoter-chloramphenical acetyl transferase (CAT) construct as an internal standard. The relative activity of each construct was measured after treatment of protoplasts for 24 h with 0.2 mM Suc and 0.3 mM phosphate (β-glucuronidase [GUS]/CAT activity). The influence of Suc (±0.2 mM Suc) and phosphate (±1.25 mM phosphate) are shown at the right and expressed as fold induction.
plast assays were useful for locating responsive domains in the VspB promoter but did not provide quantitative information about the relative activity of various promoter constructs.

The results from one set of protoplast experiments are shown in Figure 1 to document the responsiveness of each construct to various treatments. VspB constructs containing the JA response domain (−580 to −535) were much more active than constructs that lacked this part of the VspB promoter (Fig. 1, pBI232G1 versus other constructs). Constructs only containing the 50-bp JA response domain fused to the minimal (−88) 35S-promoter also showed high transcription activity in protoplasts (data not shown). In both cases, promoter activity was not increased further by addition of JA, suggesting that the process of protoplast preparation fully activated a pathway that stimulates transcription from this domain of the VspB promoter. It is unfortunate that this circumstance limited the value of further analysis of the JA response domain in protoplasts.

The −536 to −401 portion of the VspB promoter activates transcription from the truncated (−88) 35S-promoter and this activity is responsive to sugars and phosphate (Fig. 1, pBI232-1 versus pBI232). Suc stimulated promoter activity in the presence or absence of phosphate, and phosphate repressed promoter activity in the presence or absence of Suc (Fig. 1, data from the low-phosphate and high-Suc treatments are shown). This region of the VspB promoter was further divided into two sub-domains (−536 to −484 and −486 to −427) and each region was fused to the truncated (−88) 35S-promoter (pBI232-11 and pBI232-5). Each sub-domain stimulated transcription over the basal promoter indicating that sequences in each domain are able to activate transcription. Constructs containing the upstream domain (−536 to −484) were not responsive to Suc, but activity was repressed by addition of phosphate to the medium (Fig. 1, pBI232-11). In contrast, constructs containing the downstream domain (−486 to −427) were activated by Suc but not modulated by changes in phosphate (Fig. 1, pBI232-5). These and earlier results (Sadka et al., 1994) indicate that the VspB promoter contains three contiguous DNA domains that can mediate responses to JA (−585 to −536), phosphate (−536 to −484), and sugars (−486 to −427).

Identification of Protein-Binding Sites in the VspB Promoter

The location of protein-binding sites in the VspB promoter was investigated using a combination of gel mobility shift assays and DNase-I footprinting assays. The gel-shift assays shown in Figure 2 were carried out using the −611 to −451 portion of the VspB promoter as a radiolabeled probe (Fig. 2A, p26). This DNA fragment includes the JA response domain, the phosphate response domain, and a portion of the DNA domain that mediates responses to sug-
Addition of p26 to protein extracts from soybean nuclei resulted in the formation of four major DNAprotein complexes (Fig. 2B, lane 1, bands A–D). Complex formation was largely insensitive to pH (ranging from 6.4–8.8) and binding was not affected by MeJA (data not shown). Binding specificity was tested by DNase-I footprinting assays of the VspB promoter. The radiolabeled DNA fragment p26 (−611 to −451) was digested with DNase I in the presence of soybean (A) and pea (B) nuclear extracts. After digestion, DNA fragments were separated on a 5% (w/v) gel and the gel regions corresponding to the free probe (lanes marked F) and the upper bands (lanes marked B) were eluted. The purified DNAs were run on a sequencing gel along with the G+A sequencing reaction (lanes marked G+A). The boxes correspond to regions of DNA protected from digestion. Numbers indicate the nucleotide positions relative to the VspB transcriptional initiation site. C, Summary of the DNase-I footprinting results using soybean nuclear extracts. Lines above and below the sequence mark the protected regions of the upper and lower strand, respectively.

addition of unlabeled competitor DNAs to the binding mixture. The results in Figure 2B show that p26 (−611 to −451) could compete for formation of complexes A through D (lanes 4 and 5). Competitor DNAs containing sequences from −536 to −401 (p12) eliminated complexes A and B but competed to a lesser extent with formation of complexes C and D (Fig. 2B, lanes 2 and 3). This suggests that complexes C and D may involve protein binding to sequences upstream of −536 in p26. However, DNAs containing the sequence from −585 to −535 (p44, JA response domain), did not compete for binding (Fig. 2B, lanes 6 and 7). Moreover, when this region of the VspB promoter (p44) was radiolabeled and used in gel-shift assays, no specific complexes were observed (data not shown). This suggests that formation of gel-shift complexes C and D may require sequences in the JA response domain (−585 to −536) plus interaction with protein factors or sequences located downstream.

Gel-shift assays were also carried out using nuclear extracts from pea leaves to see if factors in pea would bind to the soybean VspB promoter. As shown in Figure 2C, gel-shift assays with extracts from pea nuclei were less complex than those obtained with soybean extracts and revealed two major gel-shift complexes that showed specific binding characteristics (Fig. 2C, complexes A and B). The reduction in complexity in the pea extracts was due in part to pre-incubation of the extracts with poly(dI-dC)-poly(dI-dC) for 10 min before probe addition, rather than simultaneous mixing of all binding reagents as done in Figure 2B. This change resulted in clearer gel-shift patterns presumably by reducing nonspecific binding. Therefore, this approach was adopted in subsequent DNA-binding experiments.

DNase-I footprinting assays were used to further characterize DNA-binding sites within the soybean VspB promoter. The p26 DNA (−611 to −451) was radiolabeled and incubated with protein extracts from soybean or pea nuclei followed by controlled digestion with DNase I. The partially digested complexes were subsequently separated on acrylamide gels, the shifted band with lower mobility was excised (complex A in Fig. 2), and DNA fragments were extracted and analyzed on sequencing gels. The results in Figure 3 show that four regions between −611 and −451 in the lower strand and three regions in the upper strand were protected from DNase-I digestion by soybean nuclear extracts (Fig. 3A, Boxes I–IV). Similar protected regions were identified on the upper strand of p26 using pea nuclear extracts (Fig. 3B). The footprinting results are summarized in Figure 3C. Box I was located in the JA response domain, Box II and Box III were both present in the DNA region that can mediate phosphate responses, and Box IV was located in the DNA domain that mediates sugar responses.

Further Analysis of Protein Binding to Box II

DNase-I footprinting assays revealed protein binding to Box II that is located immediately downstream from a G-box sequence (CACGTG; Fig. 4A). G-box sequences in numerous promoters are known to bind basic ZIPs in higher plants (e.g. Donald et al., 1990; Schindler et al., 1992; Shen and Ho, 1995). To test whether the G box influences protein binding to Box II, and to further investigate binding to Box II, gel-shift assays were carried out using a 32-bp oligonucleotide that spans these sequences (Fig. 4A, GAT2B). In the absence of competitor DNA, radiolabeled GAT2B formed two major complexes with proteins extracted from soybean nuclei (Fig. 4B, lane 1). Addition of unlabeled GAT2B oligo to the binding reaction eliminated complex A but not B (Fig. 4B, lanes 2 and 3). In a similar manner, a modified form of...

Figure 4. Sequences involved in protein binding to Box II. A, Oligo GAT2B corresponds to a 32-bp region of the VspB promoter that includes a G-box sequence and the Box-II sequence. Oligo AT was derived from oligo GAT2B by mutation of sequences in the G box (stars correspond to altered bases). Oligo G was derived from GAT2B through mutation of sequences in Box II. B, The −611 to −451 region of the VspB promoter was radiolabeled and used with soybean nuclear extracts to carry out gel-shift assays. Binding reactions were performed in the absence (lane 1) or presence of 50 or 100 mass excess of oligonucleotides GAT2B (lanes 2 and 3), AT (lanes 4 and 5), and G (lanes 6 and 7). The major DNA protein complexes are labeled A and B.
GAT2B containing a mutated G box (labeled AT; see Fig. 4A) also effectively competed for complex-A binding (Fig. 4B, lanes 4 and 5). In contrast, oligos containing sequence changes in Box II (Fig. 4A, labeled G) were not as effective in binding competition assays (Fig. 4B, lanes 6 and 7). These results indicate that the sequence TTAATT in Box II plays a role in protein binding and that the G-box sequence is not required for the interaction observed in gel-shift assays.

**HD-ZIPs Bind to Box II**

Genes encoding proteins that interact with the −611 to −451 portion of the VspB promoter were identified by screening a cDNA expression library from 10-d-old soybean seedlings (Vinson et al., 1988). Two of the clones isolated using this technique, GmHdl56 and GmHdl57, showed 40% sequence similarity overall, and both encoded HD-ZIPs (GenBank accession nos. AF184277 and AF184278). The sequence of GmHdl56 is shown in Figure 5A. The proteins encoded by GmHdl56 and GmHdl57 contain an N-terminal HD, followed by a ZIP, and a variable length C-terminal sequence (Fig. 5A; HD is boxed, Leu in the ZIP domain are circled). The C-terminal domains of both proteins are negatively charged and contain large numbers of Ser and Thr residues (36 out of 151 residues for GmHDL56 and 32 out of 163 residues for GmHDL57). Each of the proteins contained numerous potential recognition sequences for PKC, CK2, cAMP-dependent protein kinases, and Tyr kinases.

A comparison of the amino acid sequences of GmHDL56, GmHDL57, and HDs from other organisms is shown in Figure 5B. The six plant HDs used in this comparison share over 70% sequence identity in pairwise comparison and 30% identity when compared with the HDs of human HEX and Drosophila Antp. GmHDL56 and GmHDL57 contain 11 out of the 12 amino acids conserved in most HDs (Fig. 5B, marked with stars; Scott et al., 1989). The ZIP present in GmHDL56 and GmHDL57 is characteristic of many plant HD-ZIP proteins (Ruberti et al., 1991; Shena and Davis, 1994). The ZIP domain of the plant HD-ZIP proteins consists of up to six heptad repeats, with a Leu residue at every seventh position (Fig. 5).

DNase-I footprinting assays were performed to determine if GmHDL56 had a specific binding site on the −611 to −451 portion of the VspB promoter. A 6×-His-tagged form of GmHDL56 was prepared and purified for footprinting assays to eliminate background from other DNA-binding proteins that might be present in bacterial extracts. The results shown in Figure 6 demonstrate that GmHDL56 binds to Box II in the VspB promoter. The DNase-I footprinting results obtained with GmHDL56 were similar to those results obtained in Figure 3 using extracts of soybean nuclei.

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**Figure 5.** Soybean HD Leu zipper sequence analysis. **A.** The nucleotide and deduced amino acid sequences of GmHdl56. Boxes delimit the HD. The periodic Leu forming the ZIP are circled. **B.** Comparison of HD and ZIP sequences from different organisms. A dash indicates identity with GmHDL56. The three predicted α-helices, turn, and HD-binding domain are indicated. The 12 invariant and highly conserved amino acids in the HDs are marked with stars; Scott et al., 1989. The ZIP present in GmHDL56 and GmHDL57 is characteristic of many plant HD proteins (Ruberti et al., 1991; Shena and Davis, 1994). The ZIP domain of the plant HD-ZIP proteins consists of up to six heptad repeats, with a Leu residue at every seventh position (Fig. 5).
Figure 7 shows gel-shift assays using extracts of *E. coli* cells expressing GmHDL56 or GmHDL57. In the absence of competitor DNA, *E. coli* extracts containing either GmHDL56 or GmHDL57 form two major complexes with the $-611$ to $-451$ portion of the *VspB* promoter (Figure 7, lanes 1 and 7). Unlabeled probe DNA ($-611$ to $-451$) can compete with complex-A formation in extracts containing GmHDL56, and complexes C and D in extracts containing GmHDL57 (Figure 7, lanes 2 and 8). In contrast, a nonspecific chloroplast DNA probe did not compete significantly for binding (Fig. 7, competitor psbA, lanes 3 and 9). Oligos containing Box II and the G box and similar oligos with modified G-box sequences (GAT2B, AT) were able to compete for binding (Fig. 7, lanes 4, 5, 10, and 11). In contrast, oligo G that contains mutations in Box II (TTAATT) did not compete for binding (Fig. 7, lanes 6 and 12). These results are similar to those obtained with nuclear extracts (Fig. 4).

**DISCUSSION**

*VspB* expression is regulated during plant development and by wounding, water deficit, light, metabolites such as phosphate, carbon, and nitrogen, and by the plant regulators jasmonic acid and auxin (Mason and Mullet, 1990; Staswick, 1990; Mason et al., 1992; DeWald et al., 1994; Creelman and Mullet, 1997). This investigation focused on portions of the *VspB* promoter that respond to at least three primary agents: jasmonic acid, phosphate, and sugars. Previous studies showed that modulation of *VspB* transcription by JA, sugars, and phosphate is mediated by an approximately 185-bp DNA domain located between $-585$ and $-401$ in the *VspB* promoter. The results pre-
presented in this paper provide evidence that this promoter domain is composed of three contiguous regions that mediate responses to JA (stimulation), phosphate (repression), and sugars (stimulation).

**JA Response Domain**

Mason et al. (1993) previously demonstrated that a 50-bp DNA region of the VspB promoter, located between −585 and −535, could mediate responses to JA in vivo when fused to a truncated (−88) CaMV 35S-promoter. In the current study using protoplasts, the JA response domain alone, or part of larger segments of the VspB promoter, conferred high expression on the truncated (−88) 35S-CaMV promoter. However, constructs containing the JA response domain were not responsive to JA in protoplasts. This suggests that the JA response pathway or another parallel pathway that acts through the JA response domain is fully activated during the preparation of protoplasts. It is also possible that JA cannot mediate responses in protoplasts.

Previous comparisons of the JA response domain with other promoters, and specifically to other wound- and JA-responsive promoters, suggested that a C-rich sequence in the JA response domain might help mediate JA responses (Ryder et al., 1984; Schulze-Lefert et al., 1989; Creelman et al., 1992). In the current study, DNase-I footprinting analysis using the −611 to −451 promoter region provided preliminary evidence that proteins bind to the C-rich sequence in a region of the VspB promoter labeled Box I (ACCCTAGAACCTTC). The evidence is considered preliminary because footprints in this region were weak and only observed on one DNA strand. In addition, the JA response domain in isolation did not form stable sequence-specific gel-shift complexes, suggesting that binding to Box I may involve interaction with factors that bind outside of this domain. We reported previously that fusion of the VspB JA response domain to 35S-promoters truncated to −46 did not respond to JA, whereas fusion of this domain to 35S-promoters truncated to −88 activated transcription in the presence of JA 7-fold compared with the basal promoter (Mason et al., 1993). The −88 construct contains the as-1 cis element and binds ASF1 (Xiang et al., 1996). This complicates interpretation of the results because as-1 and other similar elements (nos-1) can mediate responses to several hormones including JA (salicylic acid, auxin, etc.; Kim et al., 1993; Xiang et al., 1996). We conclude that factors binding to the truncated (−88) 35S-promoter take the place of factors that help mediate JA responses from the VspB promoter in vivo. Further studies will be required to test if the Box-I sequence is important for JA-modulated transcription in vivo, and to identify the endogenous sequences and trans-factors that help mediate JA-induced transcription from the VspB promoter.

A G-box motif, which is characterized by the dyad sequence CACGTG, is also located in the JA response domain of the VspB promoter and other JA-responsive promoters (Bell and Mullet, 1991; Creelman et al., 1992; Kim et al., 1992; Mason et al., 1993). A large number of related basic ZIPs have been reported to bind to the G box and related sequences (i.e. Donald et al., 1990; Zhang et al., 1993; Hong et al., 1995; Lu et al., 1996). In potato (Solanum tuberosum), the G-box motif in the PinII promoter was required for JA-mediated expression (Kim et al., 1992). However, another study concluded that the G box was not required for JA-mediated responses (Lorbeth et al., 1992). In the current study, protein binding to the G box was not observed in DNase-I footprinting analysis. Therefore, additional in vivo analysis will be required to determine if the G box is involved in mediating JA or other responses of the VspB promoter.

**Sugar Response Domain**

VspB transcription is activated by sugars and repressed by phosphate. The region of the VspB promoter mediating these responses was previously localized between −536 and −401 (Sadka et al., 1994). In earlier work, it was not clear whether these metabolites acted through the same or different promoter elements. In this study, the DNA region from −536 to −484 was found to mediate responses to phosphate but not Suc, whereas the region from −486 to −427 was able to mediate responses to Suc, but not phosphate. Therefore, although addition of sugars to plant cells can alter phosphate levels through the formation of sugar phosphates, the two effectors mediate their responses through two different domains in the VspB promoter.

The sugar response domain of the VspB promoter (−486 to −427) enhanced transcription from the basal (−88) 35S-promoter in the presence of Suc. DNase-I footprinting assays of the region −611 to −451 revealed a protein-binding site, labeled Box IV, located in the sugar response domain (−474 to −488). Box IV contains the sequence GAAATAATTG that, like other sugar response elements, is AT rich (for review, see Smeeckens and Rook, 1997). Although the region from −486 to −427 can mediate responses to changing sugar levels, other portions of the VspB promoter may also be involved in this response. For example, preliminary DNase-I footprinting assays suggest that there are additional AT-rich protein-binding sites immediately downstream of −427 that may also be involved in this response (data not shown).

**Phosphate Response Domain and the Role of HD-ZIP Proteins**

The −536 to −484 domain of the VspB promoter stimulated transcription from the truncated (−88)
35S-promoter in protoplasts suspended in low-phosphate concentrations. Therefore, this domain of the VspB promoter is able to activate transcription at low phosphate, at least when combined with a truncated (~88) 35S-promoter. At high phosphate concentrations, transcription from this domain decreased nearly to basal levels. Gel-shift and DNase-I footprinting assays revealed the presence of two adjacent protein-binding sites (Box II and Box III) within the phosphate response domain. Mutation of the sequence CATTAATTAG located in Box II reduced protein binding to this domain in gel-shift assays. Trans-factor binding to Box II may help mediate inhibition of VspB transcription at high phosphate. Analysis of mutations in Box II in the context of an intact VspB promoter in transgenic plants will be needed to better define the role of this sequence in vivo.

Two soybean genes encoding HD-ZIPs were identified by screening expression libraries with the ~611 to ~451 portion of the VspB promoter. Gel-shift assays showed that E. coli extracts containing GmHDL56 or GmHDL57 formed specific complexes with the ~611 to ~451 portion of the VspB promoter. Moreover, these protein DNA complexes could be competed with oligos that contain Box II, but not by oligos containing mutated Box-II sequences. DNase-I footprinting assays using purified soybean GmHDL56 showed that this protein can protect Box II in vitro. This is consistent with the presence of HD-binding sites in Box II (core sites contain TAAT; Wolberger, 1996). The organization of TAAT sequences in Box II (TAATTAAT) is similar to the binding site for the HD protein even-skipped, where two HD proteins bind on opposite sides of the DNA (Wolberger, 1996). It is more important that the VspB Box-II sequence, CATTAATTAG, is similar to sequences previously shown to bind HD-ZIP proteins (Sessa et al., 1993; Meijer et al., 1997; Sessa et al., 1998). Studies of this class of transcription factors in Arabidopsis revealed the existence of four different groups of HD-ZIP proteins that can be distinguished in part based on their binding site specificity (Sessa et al., 1994). Box II is similar to sequences that bind to members of the first class of these proteins [HD-ZIP I; binds to CAAT(A/T) ATTG]. One member of this class of genes is activated by abscisic acid and water deficit (Soderman et al., 1996) and ectopic expression of Athb-1 alters leaf cell fate (Aoyama et al., 1995). It is interesting that a member of the second class of HD-ZIP proteins, ATHB-2, functions as a negative regulator of gene expression and is involved in mediating specific auxin responses (Steindler et al., 1999).

A rice (Oryza sativa) HD-ZIP protein of the HD-ZIP II class that binds to the sequence CAAT(G/C) ATTG also functions as a negative regulator of transcription (Meijer et al., 1997). In a similar manner, in this paper we report that HD-ZIP proteins bind to a domain of the VspB promoter that mediates reduction in transcription when phosphate levels are high. Although gel-shift and DNase-I footprinting assays demonstrate that GmHDL56/57 can bind to the VspB promoter in a sequence-specific manner, Southern analysis shows that like other genomes (Shena and Davis, 1994), soybean encodes numerous HD-ZIP proteins (data not shown). Therefore, it is not clear if GmHDL56/57 are the only HD-ZIPs that can interact with the VspB promoter in vivo. Northern analysis did not clarify this question because mRNA hybridizing to these genes is present in most tissues and developmental stages, and RNA abundance shows minimal change in response to MeJA and phosphate treatments (data not shown). Moreover, it is possible that other HD-proteins bind to Box II or Box III because both sites contain the core TAATNN sequences required to bind these proteins. Systematic examination of VspB promoter activity in plants overexpressing each HD-ZIP protein and plants with mutations in the genes encoding each HD-ZIP protein in a plant like Arabidopsis will be required to identify the specific HD-ZIP proteins involved in regulation. Even so, the identification of this class of proteins as likely candidates involved in phosphate-mediated regulation of the VspB promoter will help focus this analysis.

MATERIALS AND METHODS

Preparation of Nuclear Extracts

Soybean (Glycine max L. Merr. cv Williams 82) plants were grown in a growth chamber as previously described (Mason et al., 1992) until the seventh trifoliolate was about 1 cm long. The third and fourth trifoliates were excised under water. Individual leaflets were incubated in the light with their cut ends in 10 μM ± MeJA for 18 h. Leaf nuclear extracts were prepared as described by Jacobsen et al. (1990). Pea (Pisum sativum L. var Little Marvel) plants were grown in constant light at room temperature for 10 d. Approximately 1 kg of shoots was harvested for preparation of nuclear extracts (Green et al., 1989).

Preparation of Competitor DNA Fragments and Probes

DNA fragments of the VspB promoter (p26 [-611 to -451], p12 [-536 to -401], and p42 [four concatenated copies of -585 to -535]) were excised from vectors and gel purified. Complementary oligonucleotides (GAT2B, G, and AT) were annealed to prepare competitor DNAs. The DNA fragment, p26, was 3'-end labeled with the Klenow fragment of DNA polymerase I, gel purified, and used as a probe in gel mobility shift and DNase-I footprinting assays.

Gel Mobility Shift Assays

Binding reactions (10 μL) contained 2.5 μg of poly(dl-dC)/poly(dl-dC); 40 mM KCl; 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.5;
DNase-I Footprinting Assays

Binding reactions were scaled up 15 times from the gel mobility shift assay with the following modifications. The binding reaction also included 10 mM MgCl₂ and 1 mM CaCl₂. After the binding reaction, 0.15 units of DNase I (Boehringer Mannheim, Indianapolis) were added to the binding mixture and incubation continued for another 1.5 min. The reaction mixture was loaded onto a 5% (w/v) polyacrylamide gel. After electrophoresis, the gel was transferred to one chromatography paper (Whatman, Clifton, NJ) and exposed to a film. The exposed film was aligned with the gel and the low mobility bands and the bands corresponding to the free probe were cut out and transferred to a tube (Eppendorf Scientific, Westbury, NY). The gel slices were soaked in 1 mL of elution buffer (50 mM Tris-Cl [pH 8.0], 1% [w/v] SDS, 2 mM EDTA, and 10 ng µL⁻¹ tRNA). After boiling for 5 min, the paper shreds were removed. Another 300 µL of elution buffer was added. The tube was incubated at 55°C overnight. The supernatant was extracted once with phenol and once with CCl₄. DNA was precipitated with ethanol. Chemical sequencing reactions were performed as described by Ausubel et al. (1991). DNA samples were denatured and loaded onto an 8% (w/v) sequencing gel.

Plasmid Construction and Site-Directed Mutagenesis

The vector plasmid pBI232 was constructed by replacing the 800-bp CaMV 35S promoter of pBI221 (CLONTECH) with a minimal (−88) 35S-CaMV promoter followed by the tobacco etch virus 5’-non-translated leader sequence (Carrington and Freed, 1990). VspB promoter fragments were prepared by PCR. The PCR-amplified promoter fragments were designed with flanking restriction endonuclease recognition sequences and inserted in pBI232. Site-directed mutagenesis was performed as described by Deng and Nickoloff (1992).

Protoplast Isolation and Transient Expression Assays

Protoplasts were obtained from leaves of 4- to 6-week-old tobacco (Nicotiana tabacum cv Samsun) essentially as described by Sadka et al.(1994). Protoplasts were transfected with GUS and CAT constructs (Fromm et al., 1986) following the gene pulser electroprotocols (Bio-Rad, Richmond, CA) for Nicotiana plumbaginifolia. After electroporation, protoplasts were transferred to medium containing osmoticum (mannitol) with or without 0.2 M Suc (at constant total molarity of Suc plus mannitol), in the presence or absence of 1.25 mM phosphate (pH 7.0), and incubated for 24 h in constant light (150 µE m⁻² sec⁻¹) at 23°C. The protoplasts were divided into two parts. One part was assayed for GUS activity (Mason et al., 1993). The other part was assayed for CAT activity (Seed and Sheen, 1988).

Construction of a Soybean cDNA Expression Library in αZAP

Soybean plants were grown in growth chambers as previously described (Mason and Mullet, 1992). Total RNA was prepared from 10-d-old soybean seedlings using the method described by Chirgwin et al. (1979) and Glisin et al. (1974). Polyadenylated mRNA was isolated from total RNA using an mRNA isolation system (PolyAtract, Promega, Madison, WI). cDNA was synthesized using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). The cDNA was ligated into the Uni-ZAP XR vector and packed in vitro using Gigapack II Gold packaging extracts (Stratagene). The primary phage library contained 6 × 10⁹ recombinant plaques.

Screening of the cDNA Expression Library

The ³²P-radiolabeled DNA fragment from −611 to −451 of the VspB promoter was prepared by PCR. The PCR mixture (100 µL) contained 10 ng of the template plasmid, 1× reaction buffer, 50 µM deoxynucleotides, 50 µCi of [α-³²P]dCTP, 50 µCi of [α-³²P]dTTP, and 4 units of Taq DNA polymerase (Promega). The amplified probe was purified by passing through the G-50 column twice. The cDNA expression library was screened for proteins, which specifically interacted with the probe as described by Vinson et al. (1988).

DNA Sequence Analysis

Sequence data was generated by using a DNA sequencer (ABI 373a, Applied Biosystems Inc) with samples prepared with the ABI Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA).

DNase-I Footprinting Assays with Purified GmHDL56

The coding sequence of the GmHdl56 gene was amplified and cloned into the BamHI and KpnI site of pQE-30 (Qiagen USA, Valencia, CA). Protein expression and purification were performed using the Qiaexpress system (Qiagen). The fusion protein was isolated under denaturing conditions using a spin column (Ni-NTA, Qiagen). Binding reactions (60 µL) contained 6 µg of poly(dl-dC):poly(dl-dC), 40 mM KCl, 20 mM HEPES (pH 7.5), 0.1 mM EDTA, 5% (v/v) glycerol, 0.5 mM dithiothreitol, 10 mM MgCl₂, and 1 mM following the gene pulser electroprotocols (Bio-Rad, Richmond, CA) for Nicotiana plumbaginifolia. After electroporation, protoplasts were transferred to medium containing osmoticum (mannitol) with or without 0.2 M Suc (at constant total molarity of Suc plus mannitol), in the presence or absence of 1.25 mM phosphate (pH 7.0), and incubated for 24 h in constant light (150 µE m⁻² sec⁻¹) at 23°C. The protoplasts were divided into two parts. One part was assayed for GUS activity (Mason et al., 1993). The other part was assayed for CAT activity (Seed and Sheen, 1988).
The reactions were started by the addition of 4 μg of purified 6×-His-tagged GmHDL56, and incubated for 10 min at room temperature. DNase I (0.15 units; Boehringer Mannheim) was added to the binding reactions and incubation continued for 1.5 min. The reactions were stopped by phenol extraction. Chemical sequencing reactions were performed as described by Maxam and Gilbert (1980). DNA samples were denatured and loaded onto an 8% (w/v) sequencing gel.

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LITERATURE CITED


Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonuclease from sources enriched in ribonuclease. Biochemistry 18: 5294–5299


Donald RGK, Schindler U, Batschaer A, Cashmore AR (1990) The plant G box promoter sequence activates transcription in Saccharomyces cerevisiae and is bound in vitro by a yeast activity similar to GBF, the plant G box binding factor. EMBO J 9: 1727–1736

Feyes BJF, Benedetti CE, Penfold CN, Turner JG (1994) Arabidopsis mutants selected for resistance to the phytoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell 6: 751–759


Homeodomain Proteins Bind to the VspB Promoter


Vincon CR, LaMarco KL, Johnson PF, Landschulz WH, McKnight SL (1988) In situ detection of sequence-
specific DNA binding activity specified by a recombinant bacteriophage. Genes Dev 2: 801–806


