

A Conserved Domain of the *viviparous-1* Gene Product Enhances the DNA Binding Activity of the bZIP Protein EmBP-1 and Other Transcription Factors*

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The maize VP1 protein is a seed-specific regulator of gene expression that effects the expression of a subset of abscisic acid (ABA)-regulated genes that are expressed during the maturation program of the seed. In addition, VP1 has pleiotropic effects on seed development that are not related to ABA. In transient expression assays, VP1 has been shown to transactivate gene expression through at least two distinct promoter elements: the G boxes from the ABA-inducible wheat *Em* gene and the *SphI* box from the maize *C1* gene. We have investigated how VP1 can transactivate gene expression through diverse promoter elements by analyzing its association *in vitro* with EmBP-1, a factor that binds the *Em* promoter. We demonstrate that VP1 can greatly enhance the DNA binding activity of EmBP-1 in a gel retardation assay. This enhancing activity has also been observed on transcription factors as diverse as Opaque-2, Max, Sp1, and NF- κ B. Deletion of a small but highly conserved region (BR2) in VP1 eliminates the enhancement *in vitro* as well as the ability of VP1 to transactivate *Em* gene expression in a transient expression assay. A 40-amino acid fragment from VP1 sandwiched between the maltose-binding protein and LacZ can confer the enhancement function to this fusion protein *in vitro*. A weak and relatively nonspecific interaction between BR2 and DNA is demonstrated by UV cross-linking. The *in vitro* properties we observe for VP1 might explain the regulatory effects of VP1 on a diverse set of genes and why mutations in the *vp1* locus have pleiotropic effects.

Phytohormones such as abscisic acid (ABA)¹ are characterized by their ability to mediate a wide range of physiological responses, one of which is the expression of specific genes that are organ-, tissue-, or cell-specific (1). Such response pathways are superimposed upon the developmental regulation of gene expression. Genetic and biochemical approaches are being used to understand the mechanism by which a wide variety of hormone-responsive genes are regulated by a single hormone in a

tissue-specific manner (2, 3). For example, a set of genes that are expressed in the embryo of maturing seeds has been shown (by embryo culture and mutational studies) to require ABA (1–3). However, it is becoming clear that ABA-regulated genes are not only differentially expressed during seed development, but are differentially controlled in response to ABA in non-embryonic tissue. For example, a gene in maize (*emb5*) with strong homology to the highly conserved seed protein *Em* gene (4), as well as the globulin genes in maize (*glb1* and *glb2*) are expressed exclusively in mid-maturation embryos (5–8). Transcripts of a catalase gene (*cat1*) and members of the Rab family (*rab17* and *rab28*), however, accumulate not only in response to ABA in developing embryos, but in response to ABA in vegetative tissues as well (5, 9, 10). Although all of these genes have the common requirement for ABA for their expression in embryos, they appear to be members of different gene sets controlled by different developmental programs.

Mutations in the *viviparous-1* (*vp1*) regulatory locus in maize and its equivalent in *Arabidopsis*, *ABA-insensitive 3* (*abi3*), have pleiotropic effects during seed maturation, one of which is to control the sensitivity of cells to ABA. The *vp1* mutant fails to accumulate transcripts of the *emb5*, *glb1*, *glb2*, and other members of the class I and II gene sets (5) in the presence of ABA, whereas the *cat1* and *rab28* genes are expressed in response to ABA in *vp1* mutant embryos (10, 11). Similar to the regulation of the *emb5* gene, the homologous *Em* gene in rice (*Emp1*) and *Em1* in *Arabidopsis* require both ABA and VP1/ABI3 for expression in the embryo (12–14). Hence, *Em* and *glb* are examples of genes that require ABA and VP1 for expression in the embryo, while *cat1* and *rab28* are examples that require ABA but not VP1. The VP1/ABI3 protein, which is expressed only during seed development (6, 14), may be the factor most directly responsible for the strict regulation of genes that are expressed exclusively in seeds, e.g. *Em*. Support for this comes from the work of Parcy *et al.* (14), who demonstrated that in transgenic *Arabidopsis* plants, overexpression of ABI3 results in the ectopic expression of the endogenous *Em* gene (*AtEm1*) in vegetative tissue when exposed to ABA.

The complexity and interaction of regulatory circuits operative during seed maturation is demonstrated further by the fact that *vp1* has very pleiotropic effects during grain development, suggesting an even broader regulatory function than the ABA and embryo responses. *vp1* kernels exhibit a lack of anthocyanins in the non-embryonic aleurone tissue (15, 16), as well as reduced activities of several seed enzymes in diverse metabolic pathways (17). These data suggest that the VP1 protein may be involved in the regulation of a number of diverse genes and response pathways, only one of which is ABA perception and/or embryo-specific ABA-regulated gene expression. Although ABA is an important component of seed maturation and is essential for the expression of a subset of maturation genes, various processes and responses associated with seed maturation

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¹ The abbreviations used are: ABA, abscisic acid; bp, base pair(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MBP, maltose binding protein.

tion require factors other than ABA (18), as well as regulatory loci whose control extends beyond ABA response pathways (19). Since the *Em* gene appears to be exclusively expressed in embryos and requires both ABA and the seed-specific regulatory protein VP1, we have focused our studies toward understanding how VP1 interacts with the transcriptional controlling components of the *Em* gene.

ABA-responsive *cis*-sequences in the wheat *Em* promoter that have been identified using transient expression assays in protoplasts of rice (20, 21, 22) and maize (6)² are shown in Fig. 1A. A 76-bp segment of the *Em* promoter (Region I) has been shown to confer ABA responsiveness to the non-responsive 35S viral promoter (23). Region I includes two copies of a CACGTG element (Em1a and Em1b), which is a conserved element found in many plant (24) and other eukaryotic promoters (21), which flank an AGCAG element (Em2a) that is conserved in certain genes expressed in seeds (25). Deletion of Region I eliminates the ABA response, while mutations in either copy of the Em1 (a or b) or Em2a sequences dramatically reduces the ABA responsiveness of the *Em* promoter (23).³

Previous mobility shift assays and footprinting results with Region I demonstrated that the Em1a sequence represents a high affinity binding site for nuclear factors such as EmBP-1, a basic leucine zipper protein (bZIP) isolated from a wheat embryo cDNA library (21). Competition experiments with various Em1 elements from both regions I and II using rice nuclear extracts or bacterially expressed EmBP-1 indicates that the order of binding preference for nuclear factors is the same as for EmBP-1.⁴ A mutation in the CACGTG core of the Em1a element eliminates binding by nuclear extracts and EmBP-1 and abolishes ABA-induced expression in a rice transient assay (21).

Does VP1 interact with the *Em* promoter in the transient assay, and if so, does VP1 act through the same or different *cis* elements and *trans* factors required for the ABA response? Transient expression of the *vp1* cDNA in maize protoplasts cotransfected with the wheat or rice *Em* promoter linked to the reporter gene *GUS*, results in *GUS* expression in the absence of exogenous ABA (6, 16). In the presence of exogenous ABA, VP1 transactivation of the *Em* promoter shows a striking synergy in its transcriptional response, demonstrating that VP1 can augment the ABA signal in a transient expression system. Tetramers of either the Em1a or Em1b sequences from the wheat *Em* promoter are sufficient not only for the ABA response, but also for the VP1 transactivation and synergy between ABA and VP1.² A mutation in the CACGTG core of the Em1a element, which eliminates the ABA response and EmBP-1 binding (21), dramatically reduces the VP1 response in maize and rice protoplasts (22).² These data suggest that the Em1 elements in Region I of the *Em* promoter, and the bZIP factors which recognize them, are the sites through which the ABA and VP1 signals can elicit enhanced transcription (22).² VP1 can also transactivate through the *SphI* element in Region II of the *Em* promoter (Fig. 1A), but this is independent of the ABA response, since Region II alone cannot support an ABA response.² The NH₂-terminal region of VP1 is required for transactivation of the *Em* promoter and can serve as an acidic transcriptional activator as evidenced by its ability to be functionally replaced by the acidic domain of the *Herpes simplex* virus VP16 transcription factor (6). Sequence comparison of the maize VP1 protein (6, 26) with the product of the genetic equivalent locus

in *Arabidopsis* (*abi3*) as well as with the rice (13) and bean⁵ homologs shows three distinct regions that have extremely high amino acid sequence conservation.

In this paper, we investigate the *in vitro* interaction between recombinant VP1 and EmBP-1 proteins and the wheat *Em* promoter. We demonstrate *in vitro* that the VP1 protein enhances the binding activity of the bZIP transcription factor EmBP-1 to Em1 sequences in the *Em* promoter. The addition of VP1 to a DNA binding reaction with EmBP-1 appears to increase the effective concentration of EmBP-1 at the Em1 sites and additionally causes the formation of higher order complexes in a gel shift assay when the target sites are of low affinity. VP1 has similar enhancement effects *in vitro* on a variety of transcription factors with diverse DNA binding domains and different DNA targets. A 21-amino acid region of the VP1 protein (BR2), characterized by a basic, α -helical forming sequence with a high degree of conservation between maize, rice, bean, and *Arabidopsis*, is shown to be required for both transactivation of the *Em* promoter by VP1 in a transient protoplast gene expression assay, as well as for the *in vitro* DNA binding/enhancing activity. A 40-amino acid fragment from VP1, which includes BR2, sandwiched between the maltose-binding protein and LacZ can confer the enhancement function to this fusion protein *in vitro*. We show also a weak, relatively nonspecific DNA binding activity of the BR2 region, which we speculate may alter the conformation of DNA *in vitro*, and thus may be responsible for these more general enhancement effects.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—A *Bss*III/*Xba*I fragment of pGCF13, containing an EmBP-1 cDNA, was ligated in frame with the MBP gene of pPR997 (a preliminary version of New England Biolabs pMAL-c2 vector obtained from P. Riggs) to give plasmid pAN11. A partially filled *Bam*HI insert of pAN11 was ligated in the *Sal*I site of pPR997, yielding pAN14. This plasmid expresses the MBP fused with amino acids 217–354 of EmBP-1, which includes the bZIP domain.

For expression of the MBP-VP1 fusions, it was necessary to add *Eco*RI and *Hind*III sites next to the start and stop codons of the maize *vp1* cDNA (obtained from D. R. McCarty, University of Florida) by PCR amplification with the primers ANO-1 (GCGGAATTCATGGAAGCCTCCTC) and ANO-3 (GCGAAGCTTTTCAGATGCTCAC C). The pAN13 plasmid was then constructed by inserting this amplified product in the *Eco*RI/*Hind*III sites of pPR997. Unfortunately, the MBP-VP1 product of this plasmid turned out to be extremely unstable. Plasmid pAN15 (MBP-VP1 Δ 190), which encodes a VP1 protein that is missing the first 190 amino acids, was obtained by the removal of the *Eco*RI/*Bam*HI fragment of pAN13 and blunt ligating. We also received from Don McCarty plasmids containing the VP1 deletions Vp1–85/87 (Δ BR1) and Vp1–103/104 (Δ BR2), where amino acids 222–237 and 386–406, respectively, had been removed. *Bam*HI/*Hind*III inserts from both these plasmids were subcloned in the *Eco*RI/*Hind*III sites of pPR997. An expression plasmid encoding amino acids 372–405 of VP1 between the MBP and *lacZ* genes was constructed by inserting an *MscI*/*Pst*I fragment of the *vp1* cDNA in the *Eco*RI/*Pst*I sites of pPR997.

Finally, an *Apa*I/*Hind*III fragment, containing the maize Opaque-2 cDNA (a gift from R. J. Schmidt, University of California, San Diego), was inserted in the *Eco*RI/*Hind*III sites of pPR997 and the *Eco*RI/*Bam*HI insert of pPEXSD, encoding a rat Max cDNA (obtained from E. M. Blackwood, University of Washington), was inserted in the same sites of the expression vector. For expression of MBP-GF-14, the *Bst*UI/*Sma*I insert from a cDNA encoding a rice homolog of the maize GF-14 (Ref. 27; received from H. Uchimiya, University of Tokyo) was ligated in the *Xmn*I site of pPR997.

Purification of Fusion Proteins—Plasmids were transformed in *Escherichia coli* PR745 (New England Biolabs), and soluble protein extracts were obtained according to Edgerton and Jones (28) except that a different column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 1 mM dithiothreitol) was used and precipitation with ammonium sulfate was omitted. Fusion proteins

² Vasil, V., Marcotte, W. R., Jr., Rosenkrans, L., Cocciolone, S., Vasil, I. K., Quatrano, R. S., and McCarty, D. R. (1995) *Plant Cell* **7**, 1511–1518.

³ J. L. Magnard and R. S. Quatrano, unpublished data.

⁴ A. Hill and R. S. Quatrano, unpublished results.

⁵ A. J. Bobbs, personal communication.

were then purified using 5-ml amylose resin columns as recommended by the manufacturer (New England Biolabs). Protein concentrations were determined using a combination of the Bio-Rad protein assay and band intensity on Coomassie-stained SDS-PAGE.

Purified NF- κ B was a gift from A. S. Baldwin (University of North Carolina). SP1 was obtained from Promega.

DNA Probes—The probe for Region I has been described before (21). The fragment of the *Em* promoter that contains Region II was subcloned by purifying a 126-bp *Eco*0109I-*Eae*I fragment from pBM113Kp (20). This fragment was filled in with Klenow polymerase and ligated into the *Sma*I site of pUC118, giving rise to pAH2. To generate the Region II-labeled probe for gel shift assay, an *Eco*RI/*Bam*HI DNA fragment from pAH2 (which adds approximately 18 bp of polylinker) was purified and filled in with Klenow and [32 P]dATP. The human immunodeficiency virus type I κ B probe was provided by the Baldwin laboratory (29). The SP1 GC box was purchased from Promega and filled in with Klenow polymerase for 32 P labeling.

The following DNA oligonucleotides used in the competitive binding assay were annealed and filled in with cold nucleotide triphosphates.

Em1a: 5'-GCGCTCGAGTGCCTGGACACGTGGC-3'

5'-CGCGTCGACGTCGCGCCACGTGTC-3'

Em2a: 5'-CGCGTCCGACGACACGAGCAGGC-3'

5'-CGCCTCGAGCGTCGCGCCGATCCT-3'

Gel Shift Assay—The indicated proteins or extracts were incubated with 0.5–2 ng of probe (approximately 10,000–15,000 cpm) for 20 min at room temperature. All binding reactions included 12 mM Tris, pH 7.9, 12% glycerol, 35 mM KCl, 0.07 mM EDTA, 1 mM dithiothreitol, 7.5 mM MgCl₂. Typically, BSA (0.16 mg/ml) and poly(dI)-(dC) (600 ng to 1 μ g/reaction) were added to the binding reactions. Reactions were loaded onto a 4–5% acrylamide native gel (40:1 mono:bis ratio, 2.5% glycerol) and run in 25 mM Tris base, 190 mM glycine, and 1 mM EDTA (final pH 8.3). Electrophoresis was carried out at 10 V/cm in a cold room. When needed, Factor Xa protease digestion was performed according to the manufacturer's recommendations (New England Biolabs) on the MBP fusions separately or together (depending upon the experiment) and added to the binding reaction without prior removal of the protease.

Transient Assay in Rice Protoplasts—Protoplasts were prepared, transformed, and assayed for GUS activity as described previously (20), except that the final incubation was in Krens' F solution. For cotransformation experiments, 1 μ g each of effector and reporter DNAs was used per 1×10^6 protoplasts. The full-length Vp1 cDNA effector construct (pCR349.13s) was made by blunt end-ligating a Klenow-filled 2.0-kilobase pair *Eco*RI/*Hind*III fragment from pAN13 into a *Sma*I-digested and phosphatase-treated pDH51 (30). The Vp1 Δ BR2 construct (pAG6–20) was made by ligating a 0.8-kilobase pair *Sma*I/*Esp*I fragment from D. McCarty's Vp1–103/104 construct into a similarly digested pCR349.13s vector. The Em-GUS reporter construct (pBM207) was a derivative of pBM113Kp (20) that has one ATG translation start at the 5' end of the GUS transcript.⁶

UV Cross-linking—Annealed Region I oligonucleotides were filled-in with Klenow, dATP, dGTP, bromodeoxyuridine, and [α - 32 P]dCTP; purified on NucTrap push columns (Stratagene); and diluted to 1 ng/ μ l. In a standard cross-linking reaction, 2 μ g of fusion protein was incubated with 1 ng of labeled probe in 20 μ l of binding buffer (see above) for 20 min at room temperature. When necessary, 250 ng of unlabeled competitor was added along with the labeled probe. Afterward, the tube was opened and placed 5 cm under a 70-watt short wave UV lamp on ice for 15 min. Unbound DNA was digested by adding 1 μ l of 0.5 M CaCl₂ and 10 units of RQ1 DNase (Promega) and incubating at 37 °C for 30 min. The samples were then separated on an SDS-PAGE gel, which was subsequently Coomassie-stained, dried, and exposed to x-ray film.

RESULTS

Only Region I of the *Em* promoter can function as an independent ABA response element and in the synergistic interaction of ABA with VP1 (23).² To account for the enhanced transcription from the *Em* promoter caused by expression of *vp1* in a transient assay (6), we asked whether the VP1 protein could interact directly with the DNA in Region I (Fig. 1A) or indirectly via an interaction with EmBP-1 (Fig. 1B), the bZIP

transcription factor that binds specifically to the Em1 sequences required for the ABA and VP1 effects.

Fig. 2 demonstrates that under the conditions of our gel shift assay, a bacterially expressed truncated VP1 (VP1 Δ 190; see "Experimental Procedures" and Fig. 1B) does not directly interact with Region I. Furthermore, the use of an oligonucleotide selection assay similar to the one described by Blackwell and Weintraub (31) has failed to identify any DNA sequences that would bind to VP1 Δ 190 in a gel shift assay (data not shown). However, when recombinant VP1 Δ 190 was added to a binding reaction with EmBP-1 and a Region I probe, a striking enhancement of EmBP-1 binding activity was seen (Fig. 2). This enhancement with VP1 Δ 190 is not dependent on the maltose-binding protein since it is observed whether VP1 and EmBP-1 are present as fusions (+MBP) or after Factor Xa digestion (–MBP). The amount of added protein was kept constant in the reactions without VP1 by the addition of MBP-LacZ protein, which was purified in a manner identical to the other fusion proteins. No enhancement was observed when VP1 is replaced either with BSA or with the MBP-LacZ, MBP-GF14, and MBP-Max fusion proteins. GF14 is a protein shown to be associated with G box complexes (32), and Max is a mammalian transcription factor (33). Bannister and Kouzarides (34) have demonstrated that basic peptides can enhance the DNA binding activity of some transcription factors. The addition of positively charged poly-L-lysine to levels 50 times greater than the VP1 concentration fails to enhance the protein-DNA complex (data not shown), demonstrating that the effect of VP1 enhancement is not due to a nonspecific effect by a basic protein.

In addition to Region I, which contains high affinity binding sites for EmBP-1, Region II of the *Em* promoter was tested (Fig. 1A) for binding activity with VP1. Region II contains two ACGT elements, Em1c and Em1d, which have previously been shown to be low affinity binding sites for EmBP-1.⁷ Fig. 2 demonstrates that, like the result with the Region I probe, VP1 alone does not form a complex with the Region II probe. The addition, however, of VP1 Δ 190 to the binding reaction with EmBP-1 enhances the original complex of EmBP-1 with the region II probe and also causes the formation of a series of slower migrating complexes to form. Under these binding conditions, the higher order complexes are only apparent with the Region II probe. These same complexes can be observed on the Region II probe in the absence of VP1, when high concentrations of EmBP-1 are used in the binding reaction. These complexes may, therefore, be the consequence of additional molecules of EmBP-1 associating with the DNA probe and binding to less favorable or cryptic sites. VP1, in this case, would be increasing the apparent concentration of EmBP-1. In addition, we have noted that the enhancement of EmBP-1 by VP1 is consistently greater when the target probe bears a low affinity binding site (e.g. Region II compared to Region I; data not shown).

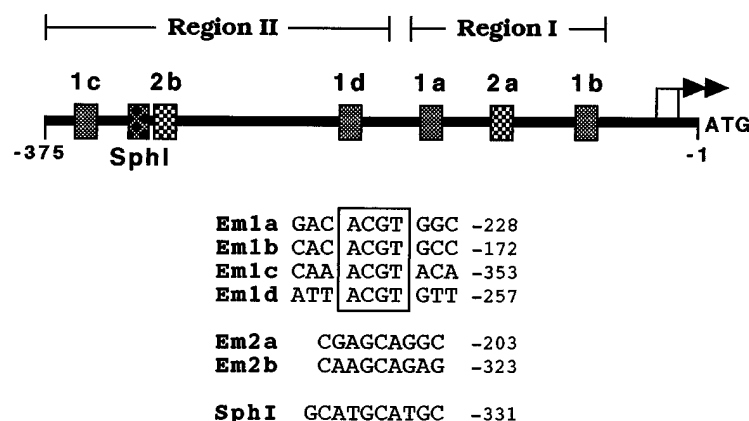
Fig. 3 demonstrates that the VP1-mediated enhancement of EmBP-1 is dependent upon the concentration of EmBP-1 in the binding reaction. The ability of VP1 to enhance was measured over a range of EmBP-1 concentrations from 0.5 to 25 nM. Maximal enhancement was seen when EmBP-1 was present at concentrations below 1 nM. This result suggests that VP1 may be helping to overcome a concentration-dependent step that limits the extent of DNA binding.

VP1 Enhances the Association of Other DNA-binding Proteins to DNA—In order to determine if the VP1 effect is specific to EmBP-1, we tested a wide variety of DNA-binding proteins including Opaque-2 (O2), a maize bZIP factor (35); Max, a rat basic helix-loop-helix factor that is known to heterodimerize

⁶ W. R. Marcotte, Jr., personal communication.

⁷ A. Hill and R. S. Quatrano, unpublished results.

A



B

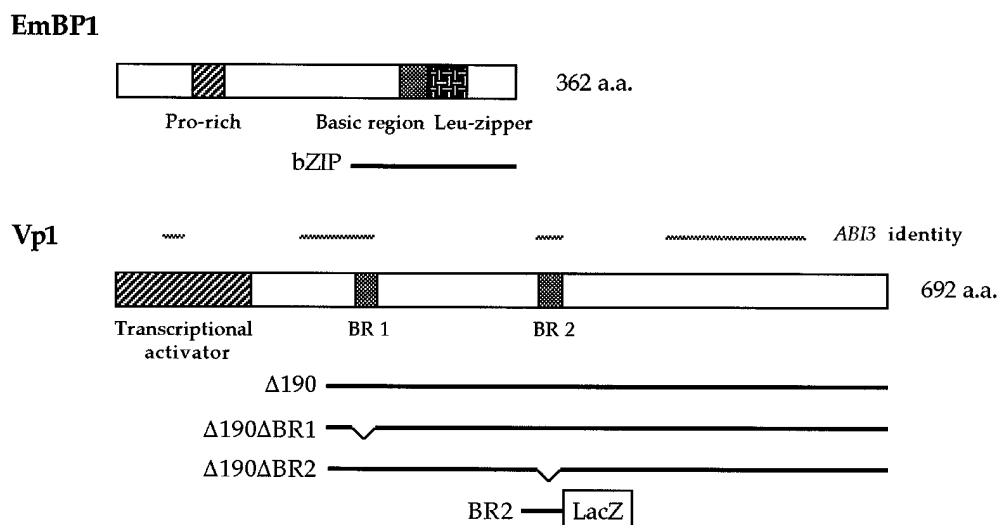


FIG. 1. **Map of the *Em* promoter and the EmBP-1 and VP1 proteins.** A, position and sequence of regulatory elements in the *Em* gene promoter. Boxes represent the Em1 (Em1a, Em1b, Em1c, and Em1d) and Em2 (Em2a and Em2b) elements and *Sph*I box identified by functional assays or by their sequence homologies to known *cis* elements. Position of these elements is given relative to the translation start site (the A of the ATG codon is coordinate 0). The arrows indicate the positions of the two putative transcriptional start sites (20, 21). Region I and Region II brackets represent the extent of the probes used in the gel shift assays. B, schematic representation of the EmBP-1 and VP1 polypeptides. In the EmBP-1 map, the shaded boxes indicate the position of the proline-rich, basic DNA binding, and leucine zipper dimerization domains. The bar below the EmBP-1 protein demonstrates that truncated bZIP retains only the basic DNA binding and leucine zipper dimerization domains. In the VP1 protein, the amino-terminal acidic domain is shown as the transcriptional activator. The position of the two basic regions BR1 (amino acids 222–237) and BR2 (amino acids 386–406) deleted from various VP1 constructs are indicated by the shaded boxes. Bars above VP1 represent regions of high homology with *ABI3*, an *Arabidopsis* homolog of VP1 (26). Bars under VP1 represent the extent of the deletion mutants expressed as maltose binding fusion proteins. $\Delta 190$ VP1 is a truncated version of VP1 that is missing the first 190 amino acids. $\Delta 190\Delta BR1$ and $\Delta 190\Delta BR2$ are derived from $\Delta 190$ VP1. The BR2-LacZ is a translational fusion of amino acids 370–409 with the COOH-terminal portion of the LacZ protein.

with Myc (33); two NF- κ B subunits homologous to the *c-rel* protooncogene (29); and the zinc finger protein SP1 (36). Both O2 and Max recognize a target sequence containing an ACGT core, which enabled us to use both Region I and Region II probes from the *Em* promoter (each containing two ACGT elements with different flanking sequences; see Fig. 1A) to test for the enhancement of these factors by VP1. The κ B target and the GC box were used in a binding reaction with the NF- κ B subunits and SP1, respectively. In order to see maximal stimulation of binding by VP1 (see Fig. 3), the concentration of DNA-binding proteins was maintained at a minimum. In some cases, this resulted in the absence of a visible complex unless VP1 was included in the binding reaction.

As seen in Fig. 4, the DNA binding activity of these widely differing transcription factors, to their own or variant target sites, can be greatly enhanced by the addition of VP1 $\Delta 190$. It is

especially interesting to note that for O2, detection of binding to the ACGT elements in Region II (Em1c and 1d), which are quite different from its preferred target, was dependent upon and enhanced by VP1. The enhancement of binding of this diverse set of transcription factors indicates that the effect of VP1 is not confined to a specific class or target site.

VP1 Increases the On-rate of DNA Binding—To determine how VP1 enhances the DNA binding activity of various DNA-binding proteins, we examined the rate of association of EmBP-1 with Region I in the absence and presence of VP1. Fig. 5 demonstrates that, in the absence of VP1, the EmBP-1 complex requires 10 min to reach maximal level. In the presence of VP1, total DNA binding was greater, as shown previously, and the complex was formed by 5 min. Thus, VP1 increases the on-rate of DNA binding.

Enhancement of EmBP-1 Binding Activity in Vitro and

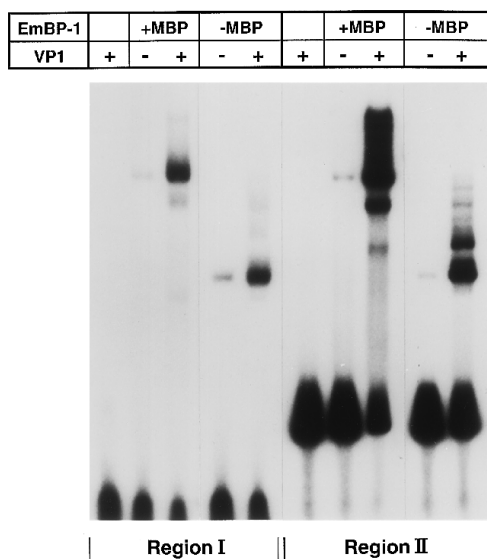


FIG. 2. **Enhancement of EmBP-1 DNA binding activity by VP1.** EmBP-1 and VP1 as fusion protein (+MBP) or digested with Factor Xa (-MBP) were assayed for DNA binding to radiolabeled Region I or Region II probes in a gel shift assay. Binding reactions contained approximately 375 ng of EmBP-1 fusion protein or 60 ng of Factor Xa-digested EmBP-1 (-MBP). Assays were carried out in the presence of either 2 μ g of MBP-LacZ (-lanes) or 2 μ g of MBP-VP1 Δ 190 (+lanes).

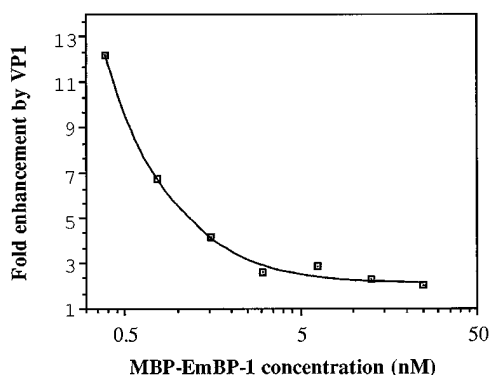


FIG. 3. **The degree of VP1-dependent enhancement is dependent upon EmBP-1 concentration.** Binding reactions were carried out with varying quantities of MBP-EmBP-1 in the presence of 1.5 μ g MBP-VP1 Δ 190 and the 32 P-labeled Em1a oligonucleotide. The amount of retarded probe was quantified using a phosphorimager and expressed as fold enhancement.

Transactivation of the Em Promoter in Vivo Is a Function of the VP1 Basic Region 2 (BR2)—We next determined the domain(s) of the VP1 protein that were essential for the observed enhancement of EmBP-1 binding. McCarty *et al.* (37) have shown genetically that the COOH-terminal 150 amino acids of VP1 are not involved in the regulation of embryo maturation by ABA. We therefore chose to focus on the upstream regions of VP1. Two highly conserved regions (BR1 and BR2; Fig. 1B) outside of the activation domain were tested for their role in the enhancement activity. Fusion proteins of VP1 Δ 190 with deletions of either BR1 (amino acids 222–237) or BR2 (amino acids 386–406) were used in the gel retardation assay with either full-length EmBP-1 or bZIP, a truncated version of EmBP-1 that retains its DNA binding and dimerization domains (see Fig. 1B). The gel shift assay in Fig. 6A demonstrates that the binding of the bZIP domain of EmBP-1 to Region I is enhanced by the addition of VP1 Δ 190. This truncated bZIP protein has retained the ability both to bind to Region I and to be enhanced by VP1. When VP1 Δ BR1 is added to the binding reaction, the same increase in binding activity of bZIP was observed,

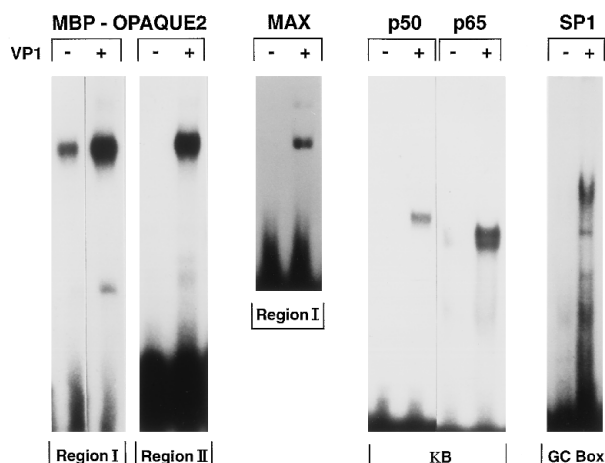


FIG. 4. **VP1 enhances the DNA binding activity of other classes of transcription factors.** Except where noted otherwise, the DNA binding activity of the following transcription factors was assayed in the presence of 2 μ g of factor Xa-treated MBP-LacZ (-lanes) or MBP-VP1 Δ 190 (+lanes). A, binding of 600 ng of purified MBP-Opaque2 to the Region I or Region II probes. B, binding of 100 ng of purified recombinant MBP-Max with the Region I probe. C, binding of recombinant NF- κ B p50 or p65 incubated with the κ B probe. D, purified Sp1 (2 ng) was added to a radiolabeled GC box probe in the presence of either 0.9 μ g of MBP-LacZ (-lane) or 0.9 μ g of MBP-VP1 Δ 190 (+lane).

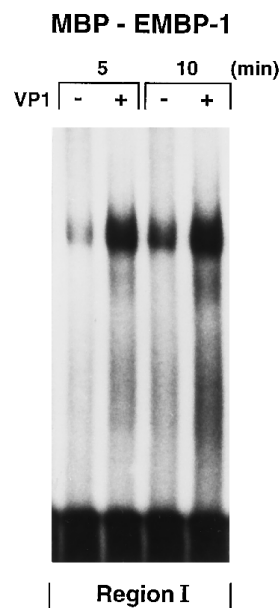
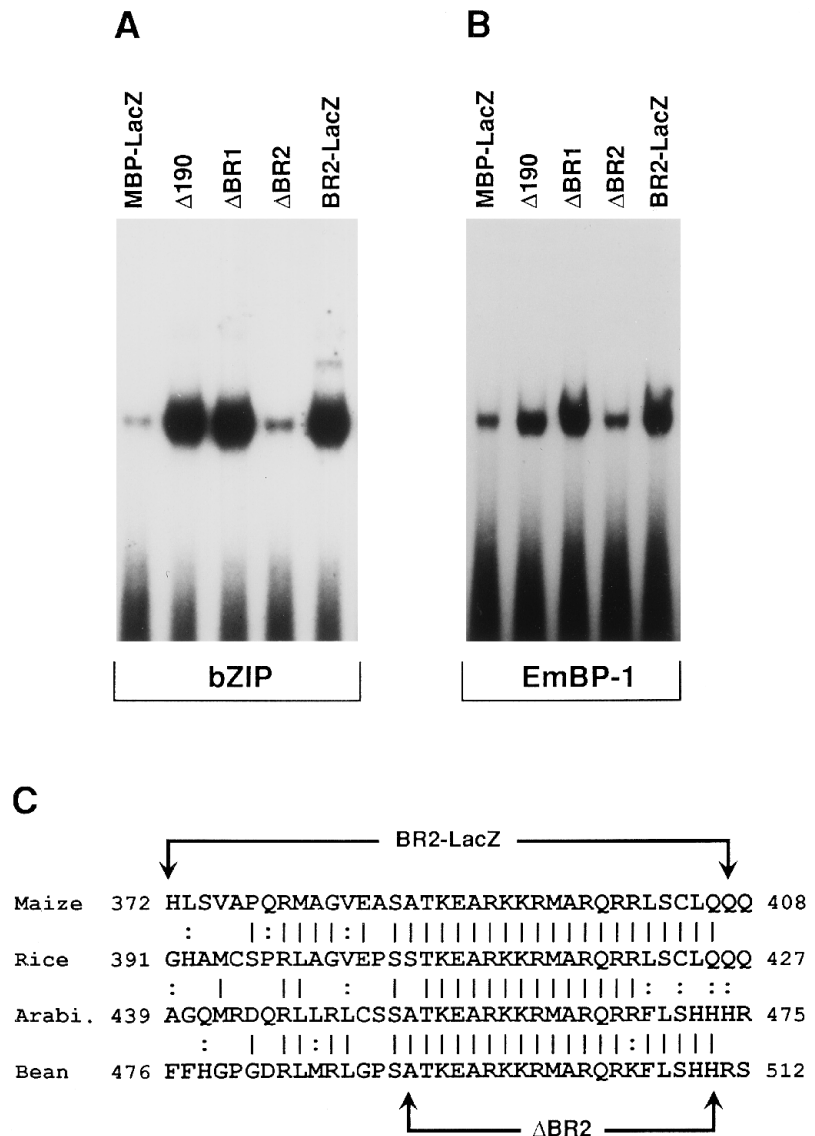


FIG. 5. **VP1 increases the rate of association of EmBP-1 with its binding site.** Binding reactions with EmBP-1 fused to MBP were carried out as in Fig. 3 (+/- VP1) except that they were incubated for 5 or 10 min at room temperature and loaded onto a gel simultaneously.

whereas, when VP1 Δ BR2 is added, there is no enhanced binding by bZIP.

To test if the BR2 region can confer the enhancement function to another protein, a fusion protein was obtained that is composed of a 40-amino acid peptide from VP1 (amino acid residues 370–409), which includes the amino acids removed in the VP1 Δ BR2 deletion, sandwiched between MBP and LacZ. When this fusion protein was tested, the binding activity of bZIP was enhanced as much as with the 502-amino acid VP1 Δ 190. Identical results were observed when the full-length EmBP-1 was used with the same series of recombinant VP1 (Fig. 6B), *i.e.* VP1 Δ 190, the VP1 Δ BR1 deletion, and the MBP-BR2-LacZ fusion protein enhanced the EmBP-1 DNA binding activity, while the VP1 Δ BR2 deletion did not. (Note that in Fig.

FIG. 6. The enhancing activity of VP1 is mediated through its BR2 domain. A, gel shift assay on the Region I probe using 20 ng of Factor Xa-treated MBP-bZIP incubated with 300 ng of either Factor Xa-treated MBP-LacZ or various VP1 deletion mutants (see Fig. 2). B, same experiment as in panel A except that 8 ng of MBP-EmBP-1 was substituted for the MBP-bZIP. C, alignment of BR2 amino acid sequence from maize with that from rice (13), *Arabidopsis* (26), and bean (see Footnote 5). The position of the BR2 sequences within the context of the full-length proteins are indicated by the coordinates at either end of the sequence. Vertical lines between the sequences indicate conserved residues, while dots indicate conservative substitutions. The bracketed region above the sequence indicates the residues used in BR2-LacZ, and the bracket below indicates the region removed in the Δ BR2 constructs.



6B the observed enhancement effect is less than that seen in Fig. 2. This can be explained by the fact that the concentration of EmBP-1 in the binding reaction is high enough to form a visible complex, in the absence of VP1. Consequently, the overall VP1-dependent enhancement is less. These results clearly demonstrate that the amino acid residues 386–406 in VP1 are necessary and sufficient for its enhancing properties and that deletion of another charged region (BR1) in another part of the VP1 protein has no effect on *in vitro* binding enhancement. Fig. 6C is an alignment demonstrating the high degree of conservation of the BR2 domain between VP1 and its homologs in rice, *Arabidopsis*, and bean.

McCarty *et al.* (6) and Vasil *et al.*² have demonstrated that VP1 expressed from a 35S promoter (and the *shrunk1* intron) transactivated different Em-GUS constructs in maize protoplasts. We constructed plasmids in which the same full-length VP1 and VP1 Δ BR2 were fused to a 35S promoter and tested for Em-GUS transactivation in our rice protoplast transient assay (23). Table I shows the results of a transient *Em-GUS* gene expression assay where full-length VP1 or VP1 Δ BR2 are co-transfected with the reporter Em-GUS. Expression of full-length VP1 resulted in a clear transactivation of *Em*, whereas expression of the BR2 deletion failed to transactivate an Em-GUS construct. Although the BR2 deletion construct has an intact activation domain (amino acids 1–122), which was

TABLE I
A full-length *Vp1* cDNA expression construct, but not a *Vp1* Δ BR2 construct, transactivates a *GUS* reporter gene driven by the *Em* promoter in transformed rice protoplasts

Treatment	Rate (\pm S.E.)
	pmol 4-MU/ μ g protein/h
No DNA	3.0 \pm 0.3
Em-GUS	77.6 \pm 19.0
Em-GUS + VP1	690.6 \pm 56.5
Em-GUS + VP1 Δ BR2	67.0 \pm 15.0

shown to be required for transactivation of the Em-GUS fusion (6), it still fails to transactivate in the rice transient assay. A ribonuclease protection assay on total RNA from replicate transfections demonstrates that full-length transcripts of the transgenes are present in the protoplasts (data not shown). These results clearly demonstrate that the amino acid residues 386–406 in VP1 are not only necessary and sufficient for the ability of VP1 to enhance the binding of EmBP-1 to DNA *in vitro*, but are required for transactivation of *Em* in an *in vivo* transient expression assay.

UV Cross-linking Shows That BR2 Can Interact with DNA—We have been unable to obtain evidence that VP1 interacts with EmBP-1 via a protein-protein interaction using immunoprecipitation, glutaraldehyde cross-linking, resin binding

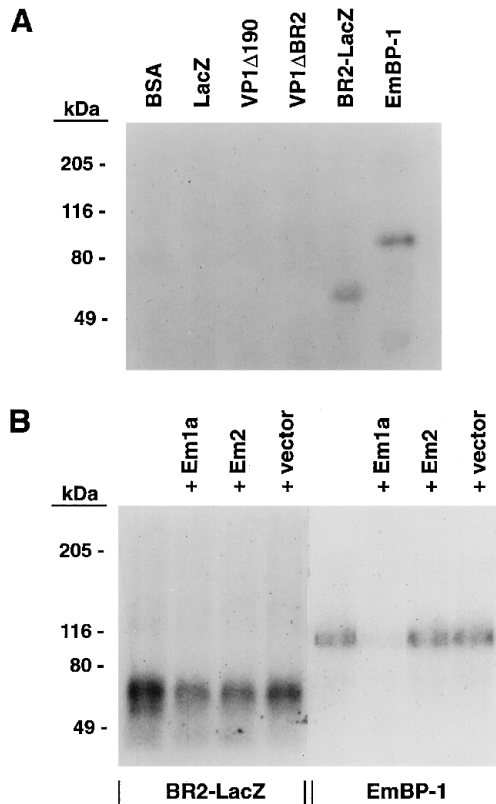


FIG. 7. DNA binding activity of the BR2 domain. A, autoradiogram of an SDS-PAGE gel after UV cross-linking of approximately 2 μ g of BSA, MBP-LacZ, MBP-VP1Δ190, MBP-VP1ΔBR2, MBP-BR2-LacZ, and MBP-EmBP-1 with a Region I probe. B, labeling of MBP-BR2-LacZ or MBP-EmBP-1 by UV cross-linking with a Region I probe. Where indicated, we added a 250-fold excess of the following competitors: Em1a, Em2a, or digested vector DNA.

assays, co-translation in a reticulocyte cell-free system, far-Western blots, and the yeast two-hybrid system. Since VP1 enhances the binding of widely different transcription factors and target sites and the effect of VP1 on EmBP-1 binding is confined to the DNA binding region (bZIP), some essential functional domain of VP1 could be interacting with DNA, thereby altering its conformation to enhance binding of sequence-specific factors.

Several recombinant proteins were incubated with a Region I probe that had been labeled with bromodeoxyuridine and [α - 32 P]dCTP and cross-linked with UV-light to capture a transient interaction. Unbound DNA was then digested with DNase and the proteins separated on SDS-PAGE. Fig. 7 shows binding (*i.e.* labeling) of MBP-BR2-LacZ and our positive control MBP-EmBP-1 to DNA. No labeling (*i.e.* binding) was observed using BSA or the MBP-fusion proteins LacZ, VP1Δ190 or VP1ΔBR2. To determine if the interaction of MBP-BR2-LacZ with DNA is specific, the cross-linking reactions were repeated in the presence of a 250-fold excess of either the Em1a element Em2 or pBluescript DNA that had been cleaved into small fragments with the restriction endonucleases *Alu*I, *Bst*UI and *Hae*III. As can be seen in Fig. 7B, binding of MBP-BR2-LacZ to region I was reduced by 40–60% with all competitors. This is in contrast to the results obtained with the specific DNA-binding factor EmBP-1, the labeling of which was totally competed only by excess Em1a.

DISCUSSION

Our results show that the bacterially expressed VP1Δ190 protein can enhance the *in vitro* DNA binding of the bZIP transcription factor EmBP-1 to its CACGTG binding sites in

Region I of the *Em* promoter. The enhancing effect on EmBP-1 binding appears to be highly selective for VP1Δ190 since a number of other (nonspecific) recombinant proteins expressed and purified in a manner similar to VP1Δ190 (*e.g.* GF14, LacZ, or Max) failed to enhance the binding of EmBP-1 to its target.

However, the ability of VP1Δ190 to enhance the binding activity of transcription factors is not limited to EmBP-1 or its preferred binding sites. The binding of O2, another member of the plant bZIP class, to ACGT sites in both Region I and Region II of the *Em* promoter is also enhanced by VP1. In addition to these members of the bZIP class, we show that VP1 also enhances the binding of members of the Rel, zinc finger, and basic helix-loop-helix class of DNA-binding proteins to their target elements, some of which are different from the ACGT sites for EmBP-1 and O2. Although dimerization is required for the bZIPs, Max, and NF- κ B to bind DNA, SP1 does not require dimerization to bind DNA (38). Hence, there is no apparent specificity of the VP1 effect with respect to class of transcription factor, target site, or requirement for dimerization.

These results raise the interesting possibility that the non-specific enhancement effect displayed by VP1 *in vitro* could be reflected *in vivo* by VP1 transactivating a number of widely differing genes through diverse promoter elements and transcription factors. This is demonstrated by the ability of VP1 to transactivate *C1*, a regulatory gene active in the control of anthocyanin biosynthesis in the non-embryonic aleurone tissue, and to transactivate the non-ABA-responsive Region II of the *Em* promoter (both require an *Sph*I element (CATGCATG) and not the CACGTG motif essential for its effect on Region I of the *Em* promoter (16)).² These activities of VP1 *in vivo* and *in vitro* are consistent with the very pleiotropic effects of VP1 during seed development (17). Hence, the enhancement effect by VP1 that we observed may be part of the mechanism by which VP1 modulates the expression of a broad range of genes and their associated diverse promoter elements and transcription factors.

However, the role of VP1 is not simply to facilitate a general increase in the expression levels of genes expressed during seed development. In maize, for example, at least two other ABA-regulated genes do not require VP1 interactions for expression in the embryo (10, 11). In *Arabidopsis*, although the *abi3-4* mutation inhibits the accumulation of numerous members of the different temporal classes of mRNA expressed during seed development, *abi3-4* does not equally affect the accumulation of all mRNAs in any one temporal class. Hence, the regulatory networks modulated by VP1/ABI3 *in vivo* is neither confined exclusively to any one temporal stage or tissue within the seed nor to all ABA-regulated genes during seed development. Perhaps the characteristic in common among the genes in the set controlled by VP1 is that they are expressed *exclusively* during seed development (*e.g.* *Em*), in part regulated by the seed-specific expression of VP1/ABI3. This is supported by the recent demonstration that overexpression of ABI3 in transgenic *Arabidopsis* results in the expression of *AtEm1* in vegetative tissue where *AtEm1* is normally not expressed (14). Whereas many other non-VP1/ABI3 regulated genes are expressed in a temporally and spatially specific pattern during seed development in response to various signals including ABA, they may also be expressed at other times during the life cycle. In any case, it is apparent that specific regulatory factors other than VP1 must be involved in the complex regulation of gene expression in seed maturation and that the nonspecific enhancing activity we see with VP1 *in vitro* must somehow be targeted to a specific subset of genes in the seed that are under VP1 regulation.

The pleiotropic nature of *vp1/abi3* in maize and *Arabidopsis*

and the absence of specificity associated with the *in vitro* enhancing phenomenon is reminiscent of properties associated with several transcriptional activators encoded by animal viruses. The Tax protein encoded by human T-cell lymphotropic virus type 1 is a transcriptional activator that can transactivate not only its own viral promoter but also a variety of cellular enhancers with diverse sequence elements. Armstrong *et al.* (39) have demonstrated, using gel shift assays, that the Tax protein has the ability to enhance the DNA binding of a variety of transcription factors from different structural classes including ATF, NF- κ B, SP1, and GAL4. This enhancement phenomenon may explain the highly pleiotropic effects of Tax on animal cells. Several *in vitro* characteristics of Tax appear to be similar to the properties of VP1 Δ 190. The Tax enhancement is not associated with a direct Tax-DNA interaction or a supershift (indicative of protein-protein interaction) in gel retardation assays. Like VP1 Δ 190, Tax has been shown to increase the on rate of bZIP proteins as well as achieve maximal enhancement of DNA binding, when the concentration of DNA-binding protein is low (40). Wagner and Green proposed that Tax works by enhancing the ability of bZIP transcription factors to dimerize via a protein-protein interaction with Tax. This mechanism does not account for the observation that Tax has been shown to associate with multiple classes of cellular enhancer-binding proteins that are not bZIP factors, including NF- κ B and SRF (41). The mechanism by which Tax enhances the DNA binding activity of such a wide range of proteins is not understood.

A different viral transcriptional activator from adenovirus, E1a, also transactivates through a number of different promoter elements. A recent report (42) presents biochemical evidence that E1a can associate with diverse transcription factor DNA binding domains, including those from AP-1, SP1, and USF. E1a appears to associate with various promoters via protein-protein interactions with a variety of transcriptional activators. However, the protein-protein association is not stable enough to survive gel electrophoresis and therefore does not result in a "supershift." Likewise, our results with VP1 and the various DNA-binding proteins in our gel shift analysis show an enhancement of the original protein-DNA complex, but no detection of a supershift that would indicate formation of a stable ternary complex. However, unlike E1a, we have been unable to detect protein-protein interaction between MBP-1 and VP1. If protein-protein interaction plays a role, it is extremely transient and we have been unable to capture it using the various approaches mentioned above.

Our efforts to identify the domain of VP1 responsible for enhancement activity, and by so doing elucidate the possible mode of action of VP1, has been guided by the work of McCarty *et al.* (15). The *vp1*-Mc allele is a transposable element insertion in the *vp1* locus causing the production of a truncated VP1 protein missing the COOH-terminal 150 amino acids. This allele specifically eliminates expression of the anthocyanin but not the maturation pathway, *i.e.* the ABA-insensitive phenotype. Hence, our efforts have been focused on the sequence of VP1 upstream of the missing COOH terminus in the *vp1*-Mc allele: in particular, two regions that are highly conserved between *Arabidopsis* and maize (BR1 and BR2). Our *in vitro* results clearly show that a 21-amino acid deletion of the BR2 domain of VP1 Δ 190 eliminates the enhancing function of VP1. That this region is also sufficient for the VP1 effect is further supported by the fact that a 40-amino acid peptide containing the BR2 region can confer full enhancing activity to MBP-LacZ.

The functional relevance of the *in vitro* effects of the BR2 domain of VP1 is supported by transient expression studies in rice protoplasts. When the deletion of BR2 from the full-length VP1 protein, is overexpressed behind a 35S promoter, a protein

results that fails to transactivate an Em-GUS construct (Table I). Although the BR2 deletion fails to activate the *Em* promoter, the NH₂-terminal acidic activation domain (amino acids 1–122) that is required for transactivation of Em-GUS (6) is present and intact. Taken together, these results demonstrate that both the NH₂-terminal acidic region and the BR2 segment are required for the VP1 function. Since BR2-LacZ lacks an activation domain, we did not detect transactivation by BR2-LacZ alone (data not shown).

Because VP1 enhances the binding of widely different transcription factors and target sites, and the effect of VP1 on MBP-1 binding is confined to the DNA binding region (bZIP), we reasoned that some essential functional domain of VP1, *e.g.* BR2, could be interacting with DNA in a relatively sequence-independent way. Such an interaction could alter the conformation of DNA (*e.g.* promote bending) to enhance binding of a number of different sequence-specific factors, such as MBP-1, O₂, etc. The BR2 domain of VP1 is a positively charged region with helix-forming potential that can form plausible DNA-binding structures. This region is highly conserved between VP1 and its homologs in *Arabidopsis*, rice, and bean (Fig. 6C). It is clear that the enhancement by VP1 Δ 190 is not associated with a stable VP1-DNA interaction, as evidenced by our inability to detect a shifted complex in a gel retardation assay. However, it appears that the BR2 domain may mediate the transient association of VP1 with DNA because of our ability to cross-link MBP-BR2-LacZ to region I. The observation that BR2 is sufficient both for the enhancing activity and DNA association suggests that a transient association of VP1 with DNA might account for its ability to enhance the binding activity of other proteins.

We are unable to explain the lack of cross-linking on MBP-VP1 Δ 190. However, one possibility is that the conformation of BR2 within the context of MBP-VP1 Δ 190 is such that its association with DNA is too transient or unstable to capture with UV cross-linking. Removing BR2 from MBP-VP1 Δ 190 and placing it within the context of a heterologous protein may serve to unmask the BR2 domain resulting in a more stable association with DNA. Another possibility is that within the context of the whole protein, the DNA is not fully protected against DNase digestion. In any case, the observation that BR2 is sufficient both for the enhancing activity and DNA association suggests that a transient association of VP1 with DNA might account for its ability to enhance the binding activity of other proteins.

It is possible that VP1 enhances the DNA binding activity of MBP-1 by transient stabilization of a local structural conformation in the CACGTG core and that binding of MBP-1 and VP1 to the same DNA is mutually exclusive. bZIP factors have recently been shown to achieve sequence-specific recognition by binding to a preorganized distorted DNA structure that constitutes the bZIP recognition target (43).

Recently it has been proposed that positively charged protein domains may contribute to protein-induced DNA bends (44). In addition, it has been shown that proteins that are known to bend DNA, for example HMG-1, are associated with enhanced binding of other DNA-binding proteins in gel retardation assays (45, 46). Future investigations will focus on whether the association of the positively charged BR2 domain with DNA results in altered DNA structure.

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REFERENCES

- Chandler, P. M., and Robertson, M. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**, 113–141
- Rock, C. D., and Quatrano, R. S. (1994) *Curr. Biol.* **4**, 1013–1015
- Rock, C. D., and Quatrano, R. S. (1995) in *Plant Hormones: Physiology, Biochemistry, and Molecular Biology* (Davies, P. J., ed) 2nd Ed., pp. 671–697, Kluwer Academic Publishing, Dordrecht, Netherlands
- Williams, B., and Tsang, A. (1991) *Plant Mol. Biol.* **16**, 919–923
- Williams, B., and Tsang, A. (1994) *Dev. Genet.* **15**, 415–424
- McCarty, D. R., Hattori, T., Carson, C. B., Vasil, V., Lazar, M., and Vasil, I. K. (1991) *Cell* **66**, 895–905
- Rivin, C. J., and Grudt, T. (1991) *Plant Physiol.* **95**, 358–365
- Paiva, R., and Kriz, A. L. (1994) *Planta* **192**, 332–339
- Scandalios, J. G. (1990) *Adv. Genet.* **28**, 1–41
- Pla, M., Gómez, J., Goday, A., and Pagès, M. (1991) *Mol. & Gen. Genet.* **230**, 394–400
- Williamson, J. D., and Scandalios, J. G. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8842–8846
- Litts, J. C., Erdman, M. B., Huang, N., Karrer, E. E., Noueiry, A., Quatrano, R. S., and Rodriguez, R. L. (1992) *Plant Mol. Biol.* **19**, 335–337
- Hattori, T., Terada, T., and Hamasuna, S. T. (1994) *Plant Mol. Biol.* **24**, 805–810
- Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M., and Giraudat, J. (1994) *Plant Cell* **6**, 1567–1582
- McCarty, D. R., Carson, C. B., Stinard, P. S., and Robertson, D. S. (1989b) *Plant Cell* **1**, 523–532
- Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L. C., McCarty, D. R., and Vasil, I. K. (1992) *Genes & Dev.* **6**, 609–618
- Dooner, H. K. (1985) *Plant Physiol.* **77**, 486–488
- Galau, G. A., Jakobsen, K. S., and Hughes, D. W. (1991) *Physiol. Plant.* **81**, 280–288
- Meinke, D. W., Franzmann, L. H., Nickle, T. C., and Yeung, E. C. (1994) *Plant Cell* **6**, 1049–1064
- Marcotte, W. R., Jr., Bayley, C. C., and Quatrano, R. S. (1988) *Nature* **335**, 454–457
- Guilinan, M. J., Marcotte, W. R., Jr., and Quatrano, R. S. (1990) *Science* **250**, 267–271
- Hattori, T., Terada, T., and Hamasuna, S. T. (1995) *Plant J.* **7**, 913–925
- Marcotte, W. R., Jr., Russell, S. H., and Quatrano, R. S. (1989) *Plant Cell* **1**, 969–976
- Foster, R., Izawa, T., and Chua, N.-H. (1994) *FASEB J.* **8**, 192–200
- Litts, J. C., Colwell, G. W., Chakerian, R. L., and Quatrano, R. S. (1991) *DNA Seq.* **1**, 263–274
- Giraudat, J., Hauge, B. M., Valon, C., Smalle, J., Parcy, F., and Goodman, H. M. (1992) *Plant Cell* **4**, 1251–1261
- Kidou, S., Umeda, M., and Uchimiya, H. (1994) *DNA Sequence* **5**, 125–129
- Edgerton, M., and Jones, A. M. (1992) *Plant Cell* **4**, 161–171
- Stein, B., Cogswell, P. C., and Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* **13**, 3964–3974
- Pietrzak M., Shillito, R. D., Hohn, T., and Potrykus, I. (1986) *Nucleic Acids Res.* **14**, 5857–5868
- Blackwell, T. K., and Weintraub, H. (1990) *Science* **250**, 1104–1110
- Lu, G., DeLisle, A. J., de Vetten, N. C., and Ferl, R. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 11490–11494
- Blackwood, E. M., and Eisenman, R. N. (1991) *Science* **251**, 1211–1217
- Bannister, A. J., and Kouzarides, T. (1992) *Nucleic Acids Res.* **20**, 3523
- Schmidt, R. J., Ketudat, M., Aukerman, M. J., and Hoschek, G. (1992) *Plant Cell* **4**, 689–700
- Kadonaga, J. T., Carner, K. R., Masiarz, F. R., and Tjian, R. (1987) *Cell* **51**, 1079–1090
- McCarty, D. R., Carson, C. B., Lazar, M., and Simonds, S. C. (1989a) *Dev. Genet.* **10**, 473–481
- Pascal E., and Tjian, R. (1991) *Genes & Dev.* **5**, 1646–1656
- Armstrong, A. P., Franklin, A. A., Uittenbogaard, M. N., Giebler, H. A., and Nyborg, J. K. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 7303–7307
- Wagner, S., and Green, M. R. (1993) *Science* **262**, 395–399
- Fujisawa, J., Hirai, H., Suzuki, T., and Yoshida, M. (1993) *Hum. Cell.* **6**, 266–272
- Liu, F., and Green, M. R. (1994) *Nature* **368**, 520–524
- Paoletta, D. N., Palmer, C. R., and Schepartz, A. (1994) *Science* **264**, 1130–1133
- Strauss, J. K., and Maher, L. J., III (1994) *Science* **266**, 1829–1834
- Onate, S. A., Prendergast, P., Wagner, J. P., Nissen, M., Reeves, R., Pettijohn, D. E., and Edwards, D. P. (1994) *Mol. Cell. Biol.* **14**, 3376–3391
- Watt, F., and Molloy, P. L. (1988) *Nucleic Acids Res.* **16**, 1471–1486