The *Viviparous-1* gene and abscisic acid activate the C1 regulatory gene for anthocyanin biosynthesis during seed maturation in maize

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The *Viviparous-1* (Vp1) gene is required for expression of the C1 regulatory gene of the anthocyanin pathway in the developing maize seed. We show that Vp1 overexpression and the hormone, abscisic acid (ABA), activate a reporter gene driven by the C1 promoter in maize protoplasts. Cis-acting sequences essential for these responses were localized. Mutation of a conserved sequence in the C1 promoter abolishes both ABA regulation and Vp1 trans-activation. An adjacent 5-bp deletion blocks ABA regulation but not Vp1 trans-activation. The latter mutant reconstructs the promoter of cl-p, an allele that is expressed during seed germination but not during seed maturation. We suggest that Vp1 activates C1 specifically during maturation by interacting with one or more ABA-regulated transcription factors.

*Key Words:* Maize, Viviparous-1 gene, C1 regulatory gene, abscisic acid

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Purple anthocyanin pigments accumulate in specific tissues of the maize seed [the scutellum of the embryo and the aleurone cell layer of the endosperm] during the maturation period of seed development. At least eight genes [A1, A2, Bz1, Bz2, C1, C2, R1, and Viviparous-1 (Vp1)] required for pigmentation of the seed are identified by mutants (Coe and Neuffer 1977). The C1, R1, and Vp1 genes have regulatory functions. The C1 protein has homology to the DNA-binding domain of the myb protooncogene [Paz-Ares et al. 1987] and contains an acidic transcriptional activation function (Goff et al. 1991). The R1 product is a helix-loop-helix protein with homology to myc [Ludwig et al. 1989]. Recent functional analyses suggest that C1 and R1 interact to activate transcription of structural genes in the anthocyanin pathway [Goff et al. 1990; Roth et al. 1991]. Whereas mutations in C1 and R1 affect only anthocyanin synthesis, the anthocyanin-less phenotype of the vpl mutant is associated with a general failure of seed maturation resulting in viviparous development of the embryo [Robertson 1955] and pleiotropic enzyme deficiencies in the aleurone [Dooner 1985]. The viviparous phenotype is correlated with a reduced sensitivity of the vpl mutant embryo to the plant hormone, abscisic acid ([ABA] Robichaud et al. 1980; Robichaud and Sussex 1986, 1987]. ABA has been widely implicated as the key hormone regulating seed maturation (Skriver and Mundy 1990).

Certain mutant alleles of vpl [vpl-McWhirter, vpl-1695, vpl-c821708, and vpl-A1] prevent anthocyanin synthesis but produce normal, nonviviparous seed [McCarty et al. 1989a], suggesting that control of the anthocyanin pathway is at least partially separable from regulation of embryo maturation. The block in anthocyanin synthesis in the vpl-R mutant is associated with failure to express the C1 gene in developing seed tissues [McCarty et al. 1989b]. This epistatic interaction suggested that Vp1 and C1 may be part of a regulatory gene hierarchy. The Vp1 block in anthocyanin synthesis is conditional [McCarty and Carson 1990]. If viviparous mutant seeds are removed from the ear prior to desiccation and allowed to continue to germinate in the light, anthocyanins accumulate in scutellum and aleurone tissues after several days. Chen and Coe [1978] described an allele of cl, called cl-p, that has an analogous effect. The cl-p seed is colorless at maturity; however, if exposed to light during development, seed tissues accumulate anthocyanins upon germination. Chen and Coe [1978] also showed that pigments accumulate in germinating [viviparous] seed of a cl-p, vpl double mutant. These observations imply that Vp1 is only required for C1 expression during seed maturation and that light can independently activate C1 and cl-p alleles during germination [McCarty and Carson 1990]. The genotype of cl-p suggests that this allele is uncoupled from developmental control.
by Vpl during maturation but remains responsive to light during germination.

In this paper we address the role of the Vpl gene in integrating control of the anthocyanin pathway into a broader program of seed maturation. We have shown recently [McCarty et al. 1991] that Vpl encodes a novel protein with properties of a transcriptional activator and that in maize protoplasts, VPl can trans-activate the promoter of Em, a downstream maturation-associated gene identified in wheat [Marcotte et al. 1988]. Here, we show that both VPl overexpression and ABA activate transcription of a reporter gene driven by C1 promoter in maize protoplasts. We present evidence that ABA regulation and VPl trans-activation require separable, as well as shared, cis-acting sequences in the C1 promoter.

Results

The C1 function is limiting for anthocyanin expression in vpl mutant tissue

We reasoned that if Vpl controlled the anthocyanin pathway by regulating the C1 or R1 genes, then constitutive expression of one or both regulatory genes in vpl mutant cells should complement the anthocyanin deficiency. Goff et al. [1990] have shown that constitutive expression of C1 and B1 [B1 being a member of the R1 gene family] from the viral cauliflower mosaic virus (CaMV) 35S promoter complements the c1 and r1 mutations, respectively, when introduced into aleurone by microprojectile bombardment. The aleurone of vpl-R mutant kernels that were otherwise homozygous for C1, R1, and all other dominant genes required for seed pigmentation was exposed by removing the pericarp. Figure 1 shows that bombardment of vpl-R aleurone tissue with 35S-C1 and 35S-B1 together or 35S-C1 alone produced many pigmented cells. Pigment was visible within 12 hr after bombardment of 17 (not shown) or 25-day postpollination aleurones. In contrast, 35S-B1 alone did not effectively complement the anthocyanin deficiency. No pigmented cells were obtained in 17-day-old materials (not shown). At 25 days, 35S-B1 bombardment produced a few pigmented cells. These cells developed pigment more slowly than C1- or C1 + B1-transformed cells, becoming visible only after a 36-hr incubation. Constitutive expression of C1 is apparently sufficient to activate the anthocyanin pathway in vpl tissue, implying that the endogenous R1 function is either already active or can be activated by C1. This experiment suggests that C1, but not R1, expression limits anthocyanin synthesis in the vpl mutant.

ABA and VPl activate the C1 promoter in maize suspension culture protoplasts

To examine the interaction of VPl with the C1 gene, a transient expression system was used to quantify C1 promoter function in maize cells under conditions where VPl expression and ABA hormone levels could be controlled. The 35S-Sh-Vp1 effector and C1-Sh-GUS reporter plasmids diagramed in Figure 2 were introduced
by electroporation into protoplasts prepared from maize suspension culture cells. The C1-Sh-GUS reporter plasmid contained 609 bp of 5'-flanking sequence from the C1 gene fused to the bacterial β-glucuronidase gene [pA gene, GUS activity]. In the 35S-Sh-Vpl plasmid, expression of the full-length VPl-coding sequence is driven by the CaMV 35S promoter. The first intron of the maize Sh1 gene was included in both gene constructs to enhance transient expression [Vasil et al. 1989]. Cotransformation with 35S-Sh-Vpl increased C1-Sh-GUS expression by about sevenfold, whereas cotransformation with a biologically neutral gene construct (35S-Sh-CAT) or a VPl antisense construct (35S-Sh-RVP) did not affect GUS activity significantly. Subsequent experiments [detailed below] showed that VPl trans-activation was dependent on specific sequences in the C1 promoter, arguing strongly that the interaction measured was promoter specific. Addition of 10 μM ABA to the culture medium after electroporation also activated C1-Sh-GUS strongly [11- to 14-fold]. However, 35S-Sh-Vpl and ABA treatments in combination did not stimulate GUS expression above the level obtained with ABA alone. We addressed the possibility that endogenous ABA syntheses or expression of the endogenous VPl gene might affect C1—GUS activation. Subculturing the maize cells in media containing 10 μM fluoridone, an inhibitor of ABA biosynthesis, prior to protoplast preparation, did not qualitatively alter ABA or VPl activation of C1-Sh-GUS (data not shown). Whereas VPl could be detected in extracts of 35S-Sh-Vpl-transformed protoplasts, any endogenous expression in untransformed protoplasts was below the limit of detection by Western blotting [data not shown]. In addition, ABA activation of C1-Sh-GUS was reduced <20% by cotransformation of protoplasts with the 35S-Sh-RVP antisense plasmid.

To further explore the interaction of VPl and ABA in regulating C1 promoter activity, we examined the ABA dose response in the presence and absence of VPl overexpression [Fig. 2C]. ABA activation of C1-Sh-GUS increased over a 0.1-100 μM concentration range. This range is in good agreement with physiologically effective ABA concentrations for arresting maize embryo devel-

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**Figure 2.** VPl overexpression and ABA activate a C1 promoter-driven GUS gene in maize protoplasts. (A) Diagrams show the structures of the 35S-Sh-Vpl, 35S-Sh-CAT, 35S-Sh-RVP effector plasmids, and the C1-Sh-GUS reporter plasmid used for electroporation experiments. The effector genes were driven by the CaMV 35S promoter with the first intron of the maize Sh1 gene included to enhance transient expression [Vasil et al. 1989]. Construction of 35S-Sh-RVP and C1-Sh-GUS is described in Materials and methods. 35S-Sh-Vpl [McCarty et al. 1991] and 35S-Sh-CAT [Vasil et al. 1989] have been described elsewhere. (B) C1-Sh-GUS was introduced into maize cell suspension culture protoplasts by electroporation alone or together with an equal amount (20 μg of DNA) of the indicated effector plasmid (see Materials and methods). After electroporation, each protoplast sample was split in two. Half was cultured in medium containing 10^{-4} M ABA, and the remainder was cultured in medium containing no ABA. GUS activity was determined 40-45 hr after electroporation. Values shown are means of three independent electroporations, with the s.e.m. indicated. Each effector was tested in a separate experiment. (C) Effect of 35S-Sh-Vpl cotransformation on the ABA dose response of C1-Sh-GUS activation. Protoplasts were electroporated with C1-Sh-GUS alone [)]) or C1-Sh-GUS in combination with 35S-Sh-Vpl [)]. Six electroporation reactions of each plasmid combination were pooled and then divided equally among media containing the indicated concentrations of ABA. Each point represents a single determination of GUS activity. Qualitatively similar results were obtained in five separate experiments.
opment (Robichaud et al. 1980). The in vivo hormone concentration in endosperm most likely falls in the lower portion of this range (Neill et al. 1986; Jones and Brenner 1987). In cells cotransformed with 35S-Sh-Vpl, a substantial component of the total GUS response (~40%) was ABA independent. Consistent with the previous experiment, the interaction of VP1 and ABA was neither synergistic nor fully additive. 35S-Sh-Vpl cotransformation did not increase expression above the maximum level obtained with ABA alone. Similar results were obtained in five independent experiments (not shown), with the exception that in some protoplast preparations, the maximum ABA-induced GUS activity obtained in the 35S-Sh-Vpl cotransformed cells was actually lower than the maximum achieved in C1-GUS-transformed cells treated with ABA alone. The basis for this apparent effect on the relative magnitude of the ABA-dependent response in some protoplast preparations is not known.

Localization of ABA and VP1 cis-responsive elements in the C1 promoter

To further localize sequences in the C1 promoter required for ABA and VP1 regulation, a series of 5’ deletions were made in the C1-Sh-GUS gene and tested in the protoplast system (Fig. 3). Mutants deleted up to position –157 relative to the transcription start remained qualitatively regulated by both ABA and VP1. Deletion of an additional 27 nucleotides to –130 abolished both ABA and VP1 activation. The –130 promoter, however, retained a significant basal activity.

Examination of the C1 promoter revealed several sequences that are potentially conserved in other ABA-regulated genes. Two sequences were detected (Fig. 4A) that resemble a putative ABA response element (ACGTGGC) identified in ABA-regulated genes of wheat (Guiltinan et al. 1989; Marcotte et al. 1989) and rice (Mundy et al. 1990). A perfect copy found in reverse orientation, at –97, was within the minimally responsive promoter defined by the terminal deletions. A second motif with one mismatch occurs at –187. To determine whether these sequences were critical for hormonal regulation, both sites were removed with small internal deletions. Although deletion of the element at –97 had a small quantitative effect on C1-Sh-GUS expression, neither mutant individually or in a double mutant combination substantially affected the ABA response relative to basal expression (Fig. 4B). A second potential conserved sequence motif (designated the Sph element) was

![Diagram](https://example.com/diagram.png)

Figure 3. Localization of C1 sequences required for ABA regulation and VP1 trans-activation in protoplasts. A series of 5’ deletions were made in the promoter of C1-Sh-GUS and tested for ABA and VP1 regulation. A] The relative ABA response of deletions up to –130. [B] The response of the same mutants to 35S-Sh-Vpl cotransformation [N.D.] not determined. [C] The ABA and VP1 response of a deletion to –157 relative to the –235 and –130 deletion mutants. s.e.m. is indicated by lines extending from bars.
VP1 activation of the C1 gene

Carty and Carson 1990). To confirm the sequence of the c1-p promoter, a 400-bp genomic fragment was amplified by polymerase chain reaction (PCR), cloned, and sequenced (Fig. 5). A 5-bp direct repeat sequence found in wild-type C1 alleles, at position -150, is present in a single copy in c1-p [Fig. 5B]. By comparison, within the 400-bp region, this was the only difference detected between c1-p and the wild-type C1 clone [Cone et al. 1986] used in this study.

A series of directed mutations were made to test sequences further in the -157 to -130 region [Fig. 6]. The Sph sequence was disrupted by making a 6-bp deletion (-136 to -131) in the -235 C1-Sh-GUS construct [Fig. 6A]. This mutation effectively abolished both ABA and VP1 activation. In addition, the sequence from -151 to -147 was deleted to reconstruct the promoter sequence of c1-p. This mutant was trans-activated by VP1, albeit less effectively than wild type. Importantly, however, it was not significantly activated by ABA. To define further the region essential for ABA and VP1 regulation, a series of multiple-base-change mutants that scanned farther in the 3' direction from -130 were tested [Fig. 6B]. Although quantitative changes in basal and induced levels of expression were evident, these mutants remained qualitatively regulated by both ABA and VP1.

Figure 5. (A) Summary of C1, cl, and c1-p phenotypes. The vp1 mutant does not prevent light-induced expression of c1-p [Chen and Coe 1978]. Similarly, light exposure will induce anthocyanin accumulation in viviparous, vp1, C1 kernels. [B] Sequence differences between C1 wild-type alleles and c1-p in the proximal 5'-flanking region. A 400-bp genomic fragment of c1-p was amplified, cloned, and sequenced (see Materials and methods). The sequence comparison shows differences that distinguish the C1-LC [top line; Paz-Ares et al. 1987], C1 [middle line, Cone et al. 1986], and c1-p alleles [bottom line]. The middle sequence was determined from the C1 allele used to construct C1-Sh-GUS. No other differences were found in the sequenced region. Additional polymorphisms occur 3' to the coding region in c1-p [Wienand et al. 1990].

Figure 4. Potentially conserved sequences identified in the C1 promoter. (A) Comparison of two sequences in the C1 5'-flanking region that resemble Em1a, a putative ABA response sequence identified by Marcotte et al. [1989], and similar sequences found in other ABA-regulated genes of maize [Vilardell et al. 1990] and rice [Mundy et al. 1990]. The location of each sequence relative to the start of transcription is indicated. (B) The Em1a-like sequence motifs in C1 are not essential for ABA regulation. Small internal deletions were made in C1-Sh-GUS(d20) that removed each of the Em1a-like sequences described in A. Plasmids carrying single deletions, as well as both deletions, were tested for ABA activation in protoplasts. (C) A series of directed mutations were made to test sequences further in the -157 to -130 region [Fig. 6]. The Sph sequence was disrupted by making a 6-bp deletion (-136 to -131) in the -235 C1-Sh-GUS construct [Fig. 6A]. This mutation effectively abolished both ABA and VP1 activation. In addition, the sequence from -151 to -147 was deleted to reconstruct the promoter sequence of c1-p. This mutant was trans-activated by VP1, albeit less effectively than wild type. Importantly, however, it was not significantly activated by ABA. To define further the region essential for ABA and VP1 regulation, a series of multiple-base-change mutants that scanned farther in the 3' direction from -130 were tested [Fig. 6B]. Although quantitative changes in basal and induced levels of expression were evident, these mutants remained qualitatively regulated by both ABA and VP1. To probe sequences still further 3', a deletion of sequences from -114 to -58 was tested [Fig. 6C]. This mutant also responded to both ABA and VP1. In summary, only mutations within the 27-bp -157 to -130 region had a qualitative affect on ABA and VP1 activation. The Sph and c1-p deletion mutants, respectively, further resolved this

| A | Em1a-like motifs |
|---|---|
| C1 (maize) | -97 TACGTGGG |
| RAB17 (maize) | -187 TACGTGGC |
| RAB16A (rice) | -205 GACGTGGC |
| EM (wheat) | -235 GACGTGGG |

| B | Sph element |
|---|---|
| C1 (maize) | -145 TCCATGCATGCAC |
| RAB17 (maize) | -258 GACGTGGC |
| RAB16A (Rice) | -79 TACGTGGC |
| EM (wheat) | -341 TACGTGGC |

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region into a 3′ region required for both responses and an upstream region required specifically for ABA activation.

Discussion
Our results indicate that VPI controls the anthocyanin pathway in the developing maize seed primarily through regulation of the C1 gene. Transient expression of C1 is sufficient to complement pigment synthesis in vpl mutant tissue. This observation suggests that C1 expression limits the anthocyanin pathway in the vpl-R mutant and that a direct interaction of VPI with the downstream structural genes in the anthocyanin pathway is not essential. Expression of the R1 regulatory gene, on the other hand, is not limiting in the vpl-R mutant. The R1 apparently does not require VPI for expression. The demonstration that overexpression of VPI in maize protoplasts activates transcription from the C1 promoter supports further the idea that VPI and C1 constitute a gene hierarchy (Fig. 7). Both VPI and C1 encode transcription factors. The C1 product has homology to the DNA-binding domain of myb (Paz-Ares et al. 1987) and contains a functional transcriptional activation domain (Goff et al. 1991). C1 and a helix-loop-helix protein encoded by members of the R1 gene family (Ludwig et al. 1989) apparently interact to activate transcription of structural genes of the anthocyanin pathway in seed tissues (Goff et al. 1990; Roth et al. 1991). Domain-switching studies have shown that VPI also contains a potent transcriptional activation domain and that this function is required for trans-activation of a target gene in maize protoplasts (McCarty et al. 1991).

VPI has been implicated in ABA hormone responses of seed tissues [Robichaud et al. 1980; McCarty et al. 1991]. Here, we have shown that activity of the C1 promoter is also regulated by ABA. The correlation between the colorless, mature seed phenotype of the c1-p allele and the failure of the c1-p-like promoter to respond to ABA in...
activated by light (Chen and Coe 1978). In a mutant vpl for the pleiotropic effects of cl-p background, wild-type is delayed until after germination when, by default, it is cl-p developmental pathway. Consequently, expression is delayed until after germination when, by default, it is activated by light (Chen and Coe 1978). In a vpl mutant background, wild-type cl and cl-p alleles have apparently similar phenotypes (delayed light-dependent pigmentation) Chen and Coe 1978; McCarty and Carson 1990, suggesting that vpl is required for abiotic activation of cl during seed development. Failure to respond to ABA would therefore apparently be the underlying basis for the pleiotropic effects of vpl on anthocyanin synthesis and maturation (Robichaud et al. 1980). In the protoplast system, VPl contributes most strongly to cl activation at low hormone concentrations (10^{-7} to 10^{-5} M ABA). This is consistent with evidence that ABA concentrations in developing maize endosperm are <1 μM [0.1-0.5 nmole/g of fresh weight (Neill et al. 1986; Jones and Brenner 1987)]. At these low concentrations, VPl action may be essential to augment ABA activation of cl.

Although overexpression of VPl in protoplasts causes activation of cl and, to some extent, cl-p promoters in the absence of ABA, it is unclear what role this hormone-independent response has in normal development. VPl alone is apparently not sufficient to activate cl-p in situ. On the other hand, hormone-independent activation of the wild-type cl allele could explain the long-standing observation that ABA-deficient mutants of maize (e.g., vp5) are generally not deficient in anthocyanins (Robertson 1955). One possibility is that the cl-p and cl promoters have different affinities for VPl and that saturating levels of VPl, not normally present in situ, partially mask this difference.

The fold activation of cl that we obtain in protoplasts with VPl (≈10-fold at 1 μM ABA) is modest compared with the level of activation (≈100-fold) of anthocyanin structural genes that has been achieved by overexpression of the cl and Rl proteins in maize tissues (Goff et al. 1990; Roth et al. 1991). Although it is possible that unknown factors limit full activation of cl in the protoplast system, the low steady-state level of cl mRNA detected in seed tissues (Paz-Ares et al. 1987; McCarty et al. 1989b) is consistent with a low rate of transcription of the cl gene in vivo. In any case, the degree of induction that we observe is probably sufficient to control the pathway given the potential for amplification downstream in the cascade afforded by the apparent sensitivity of the structural genes to activation by cl (Goff et al. 1990, 1991; Roth et al. 1991). If, for example, we assume a simple dose response curve for cl activation of the Bz1 gene that saturates over a 2 log-cycle range of protein concentration with a maximum activation of 100-fold, then a 10-fold change in cl expression could produce a 50-fold change in Bz1 expression. Higher amplification is possible if cooperativity or other synergistic interactions play a role. Moreover, C1 protein may accumulate in aleurone cells over a period of time.

The origin of the cl-p allele has interesting implications for the evolution of the maturation pathway in maize. Because cl-p occurs as a natural variant in maize populations (Chen and Coe 1978), it is unknown whether the cl- or cl-p-like promoter sequence is the ancestral form. If the cl form arose from a cl-p-like progenitor through duplication of the GTGTC sequence, it may be that the anthocyanin pathway became integrated into the maturation pathway only recently in the evolution of maize. Polymorphisms involving small direct duplications, possibly created by transposable element visitation or other mechanisms, occur frequently among alleles of other maize genes (e.g., Zack et al. 1986). Evidence for the direction of this change in the cl locus may still exist in related grasses, such as Teosinte.

A relationship between ABA regulation and VPl is suggested further by the analysis of cis-regulatory sequences in the cl promoter. Essential sequences for both responses map to a 27-bp region of the cl promoter (−157 to −130). This sequence can be resolved further into a region specifically required for ABA activation and a region required for both hormonal regulation and VPl trans-activation. A 6-bp deletion in the potentially conserved Sph element abolishes qualitative regulation by ABA and VPl without reducing basal expression. Although it is possible that this deletion affects spacing between two cis-acting elements rather than disrupting a critical sequence, mutagenesis or deletion of sequences in the 72-bp region immediately downstream failed to detect other essential sequences. Sequence motifs resembling the Sph element occur in the promoter regions of several other ABA-regulated genes isolated from cereals (Mundy et al. 1990; Marcotte et al. 1989). We have shown recently that at least one of these genes is strongly trans-activated by VPl in maize protoplasts (McCarty et al. 1991). An 8-bp CATGCATG motif, present in the core of the Sph element, is found frequently in 5’-flanking sequences of seed-specific plant genes but rarely in nonseed genes (Dickinson et al. 1988).
This sequence is also like the similarly named Sph motifs of the SV40 virus early promoter (Zenke et al. 1987).

The ABA-specific region that includes at least part of the sequence from −157 to −147 bears little obvious similarity to the Em1a element (ACGTTGGC) implicated in ABA regulation of the Em gene of wheat (Marcotte et al. 1989). The Em1a sequence is apparently essential for ABA regulation of Em in rice protoplasts (Guiltnan et al. 1990). Two sequence motifs in the C1 promoter that resemble closely the Em1a sequence are evidently not required for ABA regulation. Although both Em and C1 are trans-activated by VPl (McCarry et al. 1991), different transcription factors may mediate ABA regulation of these genes. This possibility is underscored by strikingly different interactions between VPl trans-activation and the ABA response exhibited by the Em and C1 promoters. VPl and ABA interact synergistically in activating Em (McCarty et al. 1991), whereas for the C1-driven reporter gene construct the combined response to VPl and ABA is less than additive. These features suggest that the interaction of VPl with these two genes may differ mechanistically. It is intriguing in this respect that several known vpl mutations prevent anthocyanin synthesis without blocking maturation (McCarty et al. 1989a; McCarty and Carson 1990). At least one of these mutants, vpl-McWhirter, produces a truncated protein (McCarry et al. 1989a, 1991; C.B. Carson and D.R. McCarty, unpubl.). The altered protein can apparently activate many functions associated with maturation but not C1 and the anthocyanin pathway.

The partial separation of ABA and VPl regulatory functions suggests that VPl may not be an integral component of the ABA signal transduction pathway, although it is possible that the pathway is branched and that one arm includes VPl. It is reasonable to expect that several factors may bind in the region between −157 and −130. We speculate that VPl or a complex that includes VPl may interact in this region. It is not known whether VPl binds DNA. It lacks all known conserved DNA-binding motifs (McCarty et al. 1991). Moreover, VPl expressed in Escherichia coli or by in vitro translation does not interact with oligonucleotide fragments containing the −157 to −130 region of C1 in gel-shift experiments (T. Hattori and D.R. McCarty, unpubl.). VPl could interact with this region primarily through protein−protein contacts, as has been suggested for some other transcriptional activators (e.g., Triezenberg et al. 1988). A sequence that overlaps the conserved Sp1 element is apparently required for both ABA and VPl regulation. A similar CATGCATG motif has been associated with seed-specific gene expression in plants (Dickinson et al. 1988). It seems unlikely that all genes in which this sequence has been identified are regulated by ABA or VPl. One possibility is that VPl may interact with multiple transcription factors in a way that integrates the ABA signal transduction pathway with other intrinsic developmental signals, possibly specifying tissue or positional information. Failure to achieve this integration would be consistent with the tissue-specific, hormone-insensitive phenotype of vpl.

### Materials and methods

**Transient expression in protoplasts**

Protoplasts were prepared from an embryo-derived maize suspension cell line as described previously [Vasil et al. 1989]. The cell line was derived from embryos of a DK XL80 hybrid (Dekalb Seed, Inc., Dekalb, IL). Most or all commercial hybrids are wild-type vPl and carry recessive, nonpigmenting alleles of c1 and rl. The genotype with respect to other pigment loci was not determined. Electroporation reactions included 4 × 10⁶ cells and 20 µg each of effector plasmid and reporter plasmid. Unless indicated otherwise, each treatment was replicated in triplicate. Protoplasts were cultured in growth media [Vasil et al. 1989] for 40–45 hr after electroporation and then collected by centrifugation and lysed. Soluble extracts were assayed for glucuronidase [Jefferson 1987]. GUS activities were normalized to extracted protein [Bradford 1976]. The s.e.m. for GUS activities obtained from independent electroporations done using the same protoplast preparation was typically <20% of the mean, indicating that variation in transformation efficiency within experiments was low. Because greater variation was observed among protoplast preparations made on different days, only treatment comparisons made within a single experiment were considered valid.

**Cloning and sequence analysis of a c1-p promoter fragment**

A fragment spanning the region from +16 to −390 of the c1-p gene was amplified by PCR from genomic DNA isolated from a c1-p stock (a gift of Ed Cob, University of Missouri, Columbia) size enriched for a 9.0-kbp BamHI fragment containing the gene by preparative agarose gel electrophoresis. Single-base changes were incorporated into the respective primers (5’-CCATC-GAGCTCGCTCTCG-3’ and 5’-CTGGGATCTTTGTTACTG-3’) to incorporate SstI and BamHI sites for convenient cloning into pUC19. This clone and a corresponding region of the wild-type C1 allele used in the GUS constructs (Cone et al. 1986) were sequenced by dideoxy chain termination on a DuPont Genesis 2000 DNA Analysis System (E.I. du Pont de Nemours, Inc., Wilmington, DE).

**Plasmid constructions**

All nucleic acid manipulations were carried out using standard procedures [Maniatis et al. 1982]. Restriction enzymes and DNA-modifying enzymes were used according to manufacturer’s instructions. Synthetic oligonucleotides were prepared using an Applied Biosystems DNA synthesizer and purified by either anion exchange chromatography (Mono-Q, Pharmacia, Uppsala, Sweden) or polyacrylamide gel electrophoresis.

Construction of 35S-Sh-vPl is described in McCarty et al. (1991). To construct the VPl antisense plasmid, 35S-Sh-cVP23, the cVP23 cDNA clone [McCarty et al. 1991] was cloned into the EcoRI site of pSP72 [Promega] in the orientation that placed the 5’-end proximal to the SstI site of the vector. The cVP23 fragment was cut out with EcoRV and SstI and ligated to a backbone fragment prepared from pBluescript II (Stratagene, Inc., La Jolla, CA). The fragment containing the intron was prepared by digestion with XhoI and EcoRI, and the EcoRI site was filled in by treatment with the Klenow fragment of DNA polymerase I. The
intron fragment was then ligated into 35S-RVP, which had been linearized with ClaI, blunt-ended with Klenow, and digested with XbaI.

To construct C1-Sh-GUS, a 5'-flanking fragment spanning bases -836 to +20, relative to the transcription start of the C1 gene [Paz-Ares et al. 1987], was prepared by PCR amplification from a 9-kb genomic clone of a wild-type C1 allele [Cone et al. 1986]. The primers used for the amplification were 5'-CGATCTCTCATGATGAACC-3' (sense strand) and 5'-CATAGCTCTCTGTTTCTG-3' (antisense strand). The 3' primer included a single-base change at +11 to create a unique Nhel site. The amplified product was cut with EcoRI and Nhel to generate a fragment that spanned -609 to +10. This fragment was cloned into EcoRI and Spel-digested pBluescript (I) [Stratagene, Inc.). A HindIII-XbaI fragment containing the C1 promoter was removed from this plasmid and used to replace the CaMV 35S promoter sequence in a derivative of pBl221 (Bevan 1984) in which an EcoRI site at the 3' end of the NOS terminator had been eliminated. The resulting plasmid was designated C1-GUS. An XbaI-EcoRV fragment containing the Sh1 first intron was prepared from the Bluescript subclone described above and ligated to C1-GUS, which had been digested with XbaI and Smal. This placed the Sh1 intron between the C1 promoter and the GUS-coding sequence. A slightly altered version, 35S-Sh-GUS(d00), was constructed using a Sh1 intron fragment prepared by digesting the pBluescript Sh1 subclone with EcoRI, blunting the ends with the Klenow fragment, digesting this fragment with XbaI, and inserting the intron fragment into the XbaI and Smal site of C1-GUS. Plasmid 35S-Sh-GUS(d00) was used to construct a series of 5'-deletion derivatives, d10, d20, and d30, with end points at -327, -235, and -130, respectively, using Ndel, Sau3A, and Spel sites located at these positions. To make d20, the Sau3A fragment between -235 and -78 was first cloned into the BamHI site of pUC19 so that position -235 was proximal to BamHI-Sphl. The d20 vector. By this EcoRI-Sphl site of the vector. An EcoRl-Sphl site was created in the bluescript subclone described above and ligated to C1-GUS, which had been digested with EcoRl and Sphl and the resulting fragment cloned into EcoRl and Spel-digested pBluescript(−) (Stratagene, Inc.). A HindIII-XbaI fragment containing the C1 promoter was removed from this plasmid and used to replace the CaMV 35S promoter sequence in a derivative of pBl221 (Bevan 1984) in which an EcoRI site at the 3' end of the NOS terminator had been eliminated. The resulting plasmid was designated C1-GUS. An XbaI-EcoRV fragment containing the Sh1 first intron was prepared from the Bluescript subclone described above and ligated to C1-GUS, which had been digested with XbaI and Smal. This placed the Sh1 intron between the C1 promoter and the GUS-coding sequence. 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The mutated fragment was recovered by digestion with Spel and XbaI and ligated to the Spel-XbaI backbone of d20 to generate Id2. Similar treatment of the plasmid with a new Spel site resulted in a 9-bp deletion from -184 to -177. The Sau3A-Sphl fragment of this plasmid, which spans from -235 to -135 relative to wild type, was first subcloned into the BamHI-Sphl site of pUC19 and then isolated as an EcoRI-Sphl fragment, after ligation to the EcoRI-Sphl backbone of d20, this generated Id3. The same EcoRI-Sphl fragment was ligated to the EcoRI-Sphl backbone of Id2 to generate the double mutant Id23. Id4 (a plasmid fascicle of the c-1-p deletion) was made by replacing the sequence between the Smal and Spel sites of d20 with a synthetic double-stranded oligonucleotide corresponding to a 5-bp deletion of -151 to -147. The oligonucleotides were prepared as follows: Partially complementary, synthetic oligonucleotides 5'-TACCGGGATCAGTTTTGTCGATCGATTCTTTTCGATATGCCAATTTTATACGTACCTGCTCCTTG-3' (74-mer) and 5'-AAGGTCAGCTAGGACGACACTGCTGTCTGTCGTAGCAAGGGCACCTAGTACGAGTG-3' (60-mer) were annealed, treated with the Klenow fragment of DNA polymerase I to complete both strands, and digested with Smal and Spel. The DNA fragment was purified by polycrylamide gel electrophoresis prior to ligation. Id5 was obtained by digesting d20 with Spel, treating with S1 nuclease, and circularizing with T4 ligase. This resulted in a 6-bp deletion from -136 to -131, confirmed by DNA sequencing.

To construct multiple-base-change mutants 7677, 7879, 8081, and 8283, a Stul site was introduced at position -117 in d20 to create d20(Stu). This was accomplished by PCR amplification of a fragment spanning -137 to +10 of the C1 sequence using a 5' primer that spanned the Spel site (-135) and that incorporated a single G→A base change at -117. The amplified fragment was digested with Spel and SalI, and the resulting gel-purified Spel-SalI fragment was ligated to a backbone fragment obtained by Spel and SalI digestion of d20. The 7677, 7879, 8081, and 8283 derivatives were obtained by digesting d20(Stu) with Spel and Stul and then ligating in double-stranded synthetic oligonucleotide fragments that included Spel compatible and blunt ends. The complementary pairs of oligonucleotides were as follows: For 7677, 5'-CCCTGCACGAGCAGTGCATGTT-3' and 5'-ACTTCATGATCCTGCGG-3'; for 7879, 5'-ATTCTTGAGATCCTGCGG-3' and 5'-CCGGCACTACAAAGGTG-3'; for 8081, 5'-CTCTTGAGATCCTGCGG-3' and 5'-CCGGCACTACAAAGGTG-3'; for 8283, 5'-GCTCTTACCTGCGG-3' and 5'-CCGGCACTACAAAGGTG-3'. The longer oligonucleotide of each pair was phosphorylated with T4 polynucleotide kinase prior to annealing and ligation. To construct Id6 (−114 to −58), d20(Stu) was digested with Stul and Nael and recircularized with T4 ligase.

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