Seed-Specific Gene Activation Mediated by the Cre/lox Site-Specific Recombination System

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The Cre/lox site-specific recombination system was used to activate a transgene in a tissue-specific manner. Cre-mediated activation of a β-glucuronidase marker gene, by removal of a lox-bounded blocking fragment, allowed the visualization of the activation process. By using seed-specific promoters, the timing and efficiency of gene activation could be followed within the developing tobacco (Nicotiana tabacum) embryo. To serve as a basis for analyzing gene expression after Cre-mediated activation, the timing and patterns of expression of the promoters of the genes encoding French bean (Phaseolus vulgaris) P-phaseolin and the subunit of soybean (Glycine max) P-conglycinin, as well as the transgenic tobacco embryos using the same visual marker. These seed-specific promoters were expressed earlier than anticipated. The 35S promoter was expressed earlier than the seed-specific promoters, but not in globular-stage embryos. Cre-mediated gene activation occurred approximately 1 d after promoter activation, based on developmental staging, and spread progressively throughout the embryo. The timing of gene activation was varied by altering Cre expression. Efficient Cre expression ultimately directed gene activation throughout the model tissue, whereas inefficient Cre expression resulted in mosaic tissue. Limited gene activation provides a system for cell lineage and developmental analyses.

Site-specific recombination systems derived from yeast plasmids and bacteriophage have been shown to function in plant cells. In each case the recombinase locates and interacts with its target sites, resulting in excision of DNA sequences located between directly oriented target sites or inversion of sequences located between inverted target sites. Integration between target sites on separate DNA molecules can also occur. The site-specific recombination systems expressed in plants thus far include FLP/FRT (recombinase/recognition site) of the Saccharomyces cerevisiae 2μ plasmid (Lyznizk et al., 1993; Lloyd and Davis, 1994), R/RS of the Zygosaccharomyces rouxii R1 plasmid (Onouchi et al., 1991), Gin/gix of bacteriophage Mu (Maeser and Kahmann, 1991), and Cre/lox of bacteriophage P1 (Dale and Ow, 1990; Odell et al., 1990); all systems are reviewed by Odell and Russell (1994).

The two elements of the Cre/lox system, Cre (Causes recombination) protein and loxP (locus of crossing over, P1; also lox) sites, function well as stable components of the plant genome. loxP sites integrated in the plant genome are recognized and recombined by Cre. Test genes have been designed such that Cre-mediated excision or inversion of lox-bounded DNA sequences lead to their activation or inactivation. A lox-bounded coding region placed in inverted orientation with respect to the regulating promoter was expressed after inversion (Dale and Ow, 1990). A lox-bounded fragment placed between a promoter and coding region, disrupting gene expression, was excised to restore activity (Odell et al., 1990; Bayley et al., 1992). Translocation between lox sites located on separate chromosomes also brought together a promoter and coding region, resulting in gene activation (Qin et al., 1994). Excision of an entire gene located between lox sites resulted in loss of expression (Dale and Ow, 1991; Russell et al., 1992). These alterations in the function of a test transgene occurred after the expression of a cre gene, which was introduced either by retransformation of tissue containing the lox construction or by genetic crossing of lox- and cre-containing plants. In all previous examples Cre was expressed from a relatively constitutive promoter, the 35S promoter of CaMV. Alteration in test gene expression was assayed in roots, stems, leaves, and/or germinating seedlings.

Cre-mediated gene activation in a specified tissue may provide a useful tool for developmental analyses. For example, Cre may be used to specifically activate a cell-lethal gene to determine the effects of the loss of the affected cells on surrounding tissues. Similar tissue interactions may be studied by controlling the activation of a gene whose product restores function in a mutant host. Here we examine tissue-specific regulation of gene expression mediated by Cre/lox. A silent GUS gene is activated by Cre-mediated excision of a lox-bounded fragment located between the promoter and coding region. The seed-specific promoters from the α subunit of soybean (Glycine max) β-conglycinin and from French bean (Phaseolus vulgaris) β-phaseolin were used to regulate GUS and Cre expression. These developmentally controlled systems allow an analysis of the timing and efficiency of Cre-mediated gene activation. Comparisons are made to patterns of activation induced by Cre expressed from the 35S promoter and by Cre protein that is targeted to the nucleus.

Abbreviations: Cab, Chl α/b-binding protein; CaMV, cauliflower mosaic virus; dap, days after pollination; GUS, β-glucuronidase; Nos, nopaline synthase; npt I and npt II, neomycin phosphotransferase I and II; Ocs, octopine synthase; ppb, parts per billion; SV40, simian virus 40.

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MATERIALS AND METHODS

Chimeric Gene Constructions

The French bean (Phaseolus vulgaris) β-phaseolin promoter (Ph/P) and the soybean (Glycine max) α’ subunit of β-conglycinin promoter (Cg/P; Doyle et al., 1986) were provided by J. Slightom. NcoI sites had been added at each translation initiation site while preparing the promoter fragments using the PCR technique (described by Slightom and Chee, 1991). Plasmids containing the promoter fragments also contained a 1.17-kb fragment including the phaseolin 3’ polyadenylation signal region (Ph 3’), downstream of the NcoI site. A linker was added to create the following sites between the promoters and 3’ regions: NcoI, SmaI, Asp718, and XbaI. The cre coding region was then added as an NcoI-XbaI fragment to prepare the chimeric genes, Ph/P-cre and Cg/P-cre (Fig. 1A). Each gene was transferred as a HindIII fragment into the binary vector pZS94. This plasmid contains the origin of replication and ampicillin resistance gene from pBR322, the replication and stability regions of the Pseudomonas aeruginosa plasmid pVS1 (Itoh et al., 1984), and T-DNA borders described by van den Elzen et al. (1985). To provide a plant transformation selection marker, a PstI-Asp718 fragment containing a mutant 35S-acetolactate synthase gene that confers sulfonamide resistance (described by Russell et al., 1992) was added.

A gene encoding Cre with a nuclear localization signal added at the N terminus was prepared. At the NcoI site in Cg/P-cre an oligonucleotide encoding amino acids 118 through 123 and 126 through 132 of the SV40 large T antigen (Rhys and Peters, 1989; Fig. 1A) was inserted. The oligonucleotide was designed so that the 3' NcoI site was not regenerated. The resulting Cg/P-NtCre gene was transferred as a HindIII fragment into pZS96. pZS96 is the vector pZS94 with a Nos/P-nptII-Ocs 3’ gene added to serve as a kanamycin-resistant plant selection marker.

A gene containing the 35S promoter of CaMV and the cre coding region was constructed (Fig. 1A). A promoter fragment containing the 35S promoter and leader from the Chl a/b-binding protein gene 22L (35SC; Harpster et al., 1988) was joined to the cre coding region at the NcoI site located at the translation-initiation ATG. The cre coding region was followed by a 720-bp fragment containing the polyadenylation signal sequence from the Ocs gene (Ocs 3’). An EcoRI-Asp718 fragment containing the entire 35SC-cre gene was transferred to the binary vector pZS198. pZS198 is pZS96 with the bacterial ampicillin-resistance selection marker replaced by a bacterial kanamycin-resistance selection marker (nptII; kindly provided by M. Locke).

Inactive marker GUS genes with a lox-bounded blocking fragment (lox2) between the promoter and coding region were constructed as follows (Fig. 1B). Before adding the lox2 fragment to each promoter, the NcoI site at the 3’ end of each promoter was deleted so that there would be no translation-initiating ATG 5’ to the first lox site. Plasmids containing each promoter were digested with NcoI, treated with S1 nuclease to remove the overhanging ends, and then religated (Ph/P) or cut at the SmaI site downstream of the NcoI site and religated (Cg/P). The 3’ end of each promoter fragment was sequenced to verify that the ATG from the NcoI site was removed. A linker containing XhoI and NcoI sites was added between the Asp718 and XbaI sites located 3’ to the Cg/P. The lox-polyA-lox fragment described by Odell et al. (1990) was added as a XhoI-NcoI fragment into XhoI sites located 3’ to the Cg/P. This fragment contains the polyadenylation signal sequence region from a tobacco Rubisco small subunit gene (Mazur and Chui, 1985) between directly oriented lox sites. Both lox sites are oriented so that the two ATGs present in the site are in reverse orientation with respect to the promoter. The GUS coding region (Jefferson et al., 1987) was joined, via an NcoI site at its translation start site, 3’ to lox. An Asp718-Asp718 fragment from this clone containing lox-polyA-lox-GUS was added to the Asp718 site between the Ph/P and Ph 3’, and clones with the desired fragment orientation were identified. The entire Ph/P or Cg/P-lox-polyA-lox-GUS-Ph 3’ gene was transferred as a HindIII fragment into the binary vector pZS96.

Control Ph/P-GUS and Cg/P-GUS genes were also con-
constructed (Fig. 1C). An NcoI-Asp718 fragment containing the GUS coding region was added between the NcoI and Asp718 sites between the Cg/P or Ph/P and Ph 3' in plasmids described above for the cre constructions. A HindIII fragment containing each gene was transferred into the pZS96 vector.

**Plant Transformation**

All binary vectors containing cre or lox constructions were transferred into Agrobacterium tumefaciens strain LBA4404 by three-way matings (Ditta et al., 1980), direct DNA uptake (An et al., 1988), or electroporation (Mersereau et al., 1990). Selected transformed A. tumefaciens were used to obtain Nicotiana tabacum cv Xanthi transformants as described by Odell et al. (1990) using either 300 µg/mL kanamycin or 50 ppb chlorsulfuron for selection, depending on the selection marker present.

**Seed Germination Assay**

Seed was sterilized and assayed for seedling resistance as described previously (Odell et al., 1990) using 200 mg/L kanamycin or 50 ppb chlorsulfuron.

**Cross-Pollinations**

Hand pollinations were performed as described previously (Odell et al., 1990). Initially, single-locus homozygous cre plants, identified through seed-germination assays, were crossed with primary lox2-GUS transformants that contained one or more (two or three) kanamycin-resistance loci. In this case, all seed inherit the cre gene, but 50% or less do not inherit the lox2-GUS gene. Therefore, some F1 embryos would never show GUS activation, having not inherited the GUS gene. In cases in which GUS was expressed in some F1 embryos, there were also embryos from the same cross that did not show GUS activity as expected; these were not included in figures. In cases in which no GUS activity was detected, such as in very early-stage embryos, the embryo did not show GUS activity as expected; these were not included in figures. In cases in which GUS was expressed in some F1 embryos, the embryo sample size was large enough to assure that embryos inheriting lox2-GUS would have been included in the assay.

Single-locus homozygous lox2-GUS transformants were then identified through seed-germination assays. These were crossed with primary transformants bearing 35SC-cre or Cg/ P-NTcre genes. The same inheritance situation described above occurs in the F1 seed but is reversed for the cre and lox2-GUS genes.

**Seed Dissection and Analysis**

Pods were harvested at various days after selfing (in the case of plants containing Ph/P-GUS, Cg/P-GUS, and 35S/P-GUS transgenes) or cross-pollination (dap). Seed was removed from the pod, dissected to separate the embryo from the endosperm (which often remained partially attached to the seed coat), placed directly into GUS enzyme assay buffer (Jefferson, 1987), and incubated at 37°C. Samples were examined for GUS staining at various times, and photomicrographs were taken using a Zeiss Stemi SR dissecting microscope, or a Wild M8 dissecting microscope for the higher magnifications.

**Southern Blot Analysis**

Preparation of DNA was based on the procedure of Shure et al. (1983), with modifications by J. Chen. Fresh tissue was ground in a mortar with liquid nitrogen. Buffer containing 7 M urea, 0.31 M NaCl, 50 mM Tris, pH 8.0, 20 mM EDTA, and 1% sarkosyl was added and the sample was shaken vigorously then incubated at room temperature for 10 min. The sample was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol at 25:24:1. The supernatant was passed through Miracloth, then 0.1 volume of 4.4 M NH4Ac and 0.5 volume of isopropanol were added. The DNA was spoiled, washed with 80% ethanol, dried, dissolved in water, and precipitated a final time. Approximately 10 µg of DNA was digested with Asp718 or HindIII, and the resulting fragments were separated by gel electrophoresis then transferred to a Hybond-N (Amersham) filter. Hybridization was to a digoxigenin-labeled probe, which was detected by chemiluminescence using Lumi-Phos 530 (Genius System, Boehringer Mannheim).

**RESULTS**

**Experimental Design for Seed-Specific Gene Activation**

To assess the timing and efficiency of Cre-mediated recombination in plant cells, we used the Cre/lox system to activate a marker GUS (Jefferson et al., 1987) gene in developing seed. An inactive GUS gene was constructed by placing a lox2-bounded DNA fragment (represented by lox2), containing a polyadenylation signal sequence, between the promoter and coding region (Fig. 1B). The directly repeated lox sites provide the substrate for Cre-mediated excision of the blocking fragment, leading to expression of GUS (Fig. 1D). Cre-mediated recombination of the lox2-GUS gene in vitro did give the expected products. This activatable marker and cre were regulated by two seed-specific promoters, derived from genes encoding French bean β-phaseolin (referred to as β-phaseolin promoter and Ph/P) and the soybean α subunit of β-conglycinin (referred to as α'-β-conglycinin promoter and Cg/P). When this work was initiated, the patterns of expression of these two promoters in regions of the embryo and in the endosperm of transgenic tobacco seed were not well established, and we expected different patterns for their expression. Control GUS genes were constructed without the intervening lox2-bounded fragment (Fig. 1C). For comparison, cre was also expressed from the CaMV 35S promoter. The timing of marker gene expression after activation and the extent to which it is activated throughout the embryo were determined. These analyses characterize the Cre/lox recombination process in plant cells.

**Preparation of Transgenic Plants**

Each lox-inactive chimeric GUS gene and control GUS gene was stably integrated into the tobacco genome through A. tumefaciens-mediated transformation. A linked nptII gene, conferring kanamycin resistance, was used to select the transformants and to identify lines carrying a single integrated locus. Lines were screened for GUS activity in mature seed. Of 12 kanamycin-resistant transformants with Ph/P-GUS or...
Cg/P-GUS in the introduced T-DNA, only 2 lacked GUS activity in mature seed. In these 2 lines an intact GUS gene may not have been integrated. Of 15 kanamycin-resistant lines transformed with a GUS gene interrupted by the lox-bounded fragment, 8 showed no GUS activity, whereas 7 expressed a very low level of GUS activity in mature seed relative to the Ph/P-GUS and Cg/P-GUS levels. Overnight incubation in GUS assay buffer was required to detect light blue staining, whereas staining began to appear within 15 min in seed with no blocking fragment. In the 8 negative lines the intervening lox-bounded fragment appeared to be effective in blocking expression of the GUS coding region, although it is possible that a few of them did not integrate an intact GUS gene. Seven of these lines were tested for Cre activation of GUS as described below. In all but 1, GUS expression could be activated, verifying the presence of the intact gene. The blocking fragment was not completely effective, as evidenced by the 7 lines with low GUS activity. These were not examined to determine whether the "inactive" GUS gene construction was transferred in an intact form.

Separate tobacco lines carrying chimeric seed promoter-cre genes were selected using a sulfonylurea-resistant acetolactate synthase marker. By seed-germination assays on chlorosulfuron, four single-locus lines were identified, then homozygotes in these lines were identified. Of these four lines, three were able to activate the inactive GUS gene as described below. In the fourth line an active cre transgene may not have been integrated.

In the course of these experiments, two different gene constructions containing the 35S promoter and cre coding region were used. Homozygous tobacco plants with a 35S-cre transgene were produced and assayed in previous studies (cre/Hpt-A, Odell et al., 1990; Russell et al., 1992). This chimeric gene has an untranslated leader composed of sequence from the leader of the 35S RNA of CaMV (21 bp), and sequence upstream of the translation start site of the bacteriophage P1 cre gene (about 50 bp). In a second gene, called 35SC-cre, the leader of the petunia Cab gene follows the 35S promoter, and is joined to the start of the cre coding region by an Ncol site (see "Materials and Methods"). Tobacco transformants containing the 35SC-cre transgene were selected using a linked kanamycin-resistance selection marker. Other control plants bearing a GUS gene expressed from the same 35S promoter linked to the Cab leader (35SC-GUS; Fig. 1C) were produced and described previously (Russell et al., 1992).

Expression of Ph/P, Cg/P, and 35SC/P All Initiate at the Heart Stage

Previously, expression of the β-phaseolin, α' β-conglycinin, and 35S promoters in tobacco embryos and endosperm. All embryos are shown at the same magnification except for those in F, bottom row, and G, as indicated below. All samples were incubated in GUS assay buffer overnight at 37°C unless otherwise specified. A, Embryos containing the Ph/P-GUS gene dissected 10 dap. B, Endosperm of seed containing Cg/P-GUS dissected 10 dap. C, Embryos containing Cg/P-GUS dissected 9 dap. D, Embryos containing Cg/P-GUS dissected 11 dap and incubated for 2 h in GUS assay buffer. E, The same embryos as in D incubated overnight. F, Embryos containing 35SC-GUS dissected 10 dap. The upper set is shown at the same magnification as those in A-E. The lower set contains the same embryos shown at 1.6X higher magnification for better visualization. G, Embryos containing 35SC-GUS dissected at 10 dap and incubated in GUS assay buffer for 2 h, shown at 1.6X higher magnification than those in A to E.
Figure 3. GUS activity following Cre-mediated activation of lox²-GUS genes. All samples are from F₁ seed unless otherwise specified. A, Embryos inheriting Cg/P-lox²-GUS and Cg/P-cre dissected 14 dap. Inset, Embryos inheriting Ph/P-lox²-GUS and Ph/P-cre dissected 12 dap. B, Endosperm of seed inheriting Ph/P-lox²-GUS and Ph/P-cre dissected 11 dap. C, Embryos inheriting Ph/P-lox²-GUS and 35S-cre dissected at 13 dap. Inset, Embryos inheriting Ph/P-lox²-GUS and 35S-cre dissected at 12 dap. Note that these embryos are inverted. D, Endosperm of seed inheriting Ph/P-lox²-GUS and 35S-cre dissected at 16 dap. E, Embryos inheriting Cg/P-lox²-GUS and 35S-cre dissected at 12 dap. F, F₂ embryos from F₁ plants grown from F₁ seed that inherited Ph/P-lox²-GUS and Ph/P-cre dissected 10 dap.

describe stages of embryo development than dap. In Figures 2 and 3, embryos are shown at the same magnification (except in Fig. 2, F and G) so that the stages of development can be compared by the size and shape of the embryos. Those in Figure 2A are entering the heart stage or are hearts or young torpedos. Figure 3A includes embryos of torpedo and early to maturing cotyledon stages. Embryos in the bottom row of Figure 2, F and G, and Figure 4 are shown at higher magnification for better visualization.

The expression patterns of the β-phaseolin and α' β-conglycinin promoters were similar throughout seed development. No GUS activity was observed in globular-stage embryos. As embryos entered the heart stage, spots of GUS activity were observed just below the cotyledonary lobes (see
More spots accumulated in this area, creating a ring of GUS activity in the transition zone between the developing cotyledons and embryo axis of heart-stage embryos. This ring of GUS activity was observed in the three independent transformants bearing the 35SC-GUS gene that were analyzed. Surprisingly, GUS activity was also absent in globular embryos. These unstained globular embryos are not shown due to the difficulty of photographing them under the conditions we employed. GUS activity appeared and spread more rapidly than when expressed by the phaseolin and conglycinin promoters. Embryos entering the heart stage showed a patch of GUS activity in the top central region (Fig. 2F). The expression spread rapidly; mature heart-stage embryos stained entirely blue. This timing varied slightly between independent transformants, but was always substantially earlier than observed for the phaseolin and conglycinin promoters. GUS activity also developed in the endosperm. After a short incubation in GUS assay buffer, the tips of the cotyledons and the lower part of the axis remained unstained, indicating that these are the regions with the weakest promoter expression (Fig. 2G).

The embryo expression of both promoters was much earlier than is generally described for seed storage proteins. Therefore, eight independent transformants containing Ph/P-GUS and four containing Cg/P-GUS were assayed. All but one showed strong endosperm expression and similar patterns of embryo expression. In several lines the timing varied slightly, being either a bit more rapid or delayed than that described above. One exceptional Ph/P-GUS line showed much reduced expression; spots of GUS activity appeared first in torpedo stages, which stained entirely blue in all of the other lines (data not shown). Mature embryos of this line still did not stain solidly blue, but were blotchy, indicating reduced GUS expression. This pattern could be explained by greatly reduced functioning of the phaseolin promoter, due to its position of integration in the tobacco genome in this particular line.

Incubation of embryos in GUS assay buffer for a short period of time allows the localization of regions with strongest GUS expression. The cotyledon-axis junction region is always first to stain for GUS activity, even in embryos and later-stage embryos that stain entirely blue during longer incubations (Fig. 2, D and E). Thus, this region, which is the first to show phaseolin and conglycinin promoter expression at the heart stage, maintains the highest level of expression throughout later stages of development.

The 35SC promoter showed a different pattern of expression, observed in the three independent transformants bearing the 35SC-GUS gene that were analyzed. Surprisingly, GUS activity was also absent in globular embryos. These unstained globular embryos are not shown due to the difficulty of photographing them under the conditions we employed. GUS activity appeared and spread more rapidly than when expressed by the phaseolin and conglycinin promoters. Embryos entering the heart stage showed a patch of GUS activity in the top central region (Fig. 2F). The expression spread rapidly; mature heart-stage embryos stained entirely blue. This timing varied slightly between independent transformants, but was always substantially earlier than observed for the phaseolin and conglycinin promoters. GUS activity also developed in the endosperm. After a short incubation in GUS assay buffer, the tips of the cotyledons and the lower part of the axis remained unstained, indicating that these are the regions with the weakest promoter expression (Fig. 2G).

The short incubation also allows the detection of a spot of strong GUS activity at the tip of the root axis, a pattern not observed in the phaseolin promoters.

**Cre-Mediated Gene Activation**

Once we determined the timing and pattern of GUS expression directed by the phaseolin and conglycinin promoters in individual embryos, we could compare it to the GUS expression seen after Cre-mediated activation of the loxP-GUS gene. Cre and lox elements were joined in F1 seed
by crossing a plant containing a cre gene and a plant containing a lox-GUS gene. Seeds of varying developmental stages were dissected and the embryos and endosperm were stained for GUS enzyme activity. GUS activity was observed in embryos and endosperm of seed derived from crosses between lox and cre plants, indicating that the lox-GUS gene was activated, whereas seed from crosses between lox plants and control wild-type plants never showed any GUS staining. Activation of the lox2-GUS gene by Cre-mediated recombination, diagrammed in Figure 1D, was later verified in F1 plants. DNA of cre $\times$ lox F1 plants analyzed on Southern blots showed GUS probe-hybridizing bands of the sizes that would be expected after excision of the DNA segment between the lox sites (Fig. 4).

Similar timing and patterns of GUS activation were observed in seed derived from crossing four independent Ph/P-Lox2-GUS transformants with a Ph/P-cre plant, and two independent Cg/P-Lox2-GUS transformants with two independent Cg/P-Cre transformants (all four combinations). In each case no GUS activity was observed in heart-stage or young torpedo-stage embryos. As described above, we unexpectedly found that the $\beta$-phaseolin and $\alpha'$-$\beta$-conglycinin promoters are active during these stages. It was critical to visualize this early expression of these promoters to determine that a delay in expression occurred when recombination between lox sites was required to activate the GUS gene. The first few spots of GUS activity appeared in torpedo-stage embryos (Fig. 3A). This represents a delay of about 1 d in the developmental time course. Spots of GUS activity occurred first at the cotyledon-axis junction, which is the region of earliest and highest $\beta$-phaseolin and $\alpha'$-$\beta$-conglycinin promoter expression (see Fig. 2, A–D). The GUS-expressing region then spread up into the cotyledons and down into the axis, until the whole maturing cotyledon-stage embryo stained blue. Activation occurred coordinately in the endosperm, initially in spots of activity that then spread throughout the endosperm (Fig. 3B). No difference in the timing of GUS gene activation was observed in F1 embryos derived from reciprocal crosses (data not shown).

Eliminating the Delay in Gene Activation

When the same developmentally regulated promoters direct expression of Cre and GUS, the time that is required for Cre expression and the recombination process itself is seen as a delay in GUS expression. Earlier expression of Cre could eliminate this delay. We initially thought that the 35S promoter would be expressed throughout embryo development in a more constant manner than are the seed-specific promoters. If this were true, Cre protein would be present and able to create the activated GUS gene prior to the time when the seed-specific promoters are expressed. Therefore, GUS activity would appear with the same timing as the expression of Ph/P-GUS. Surprisingly, this was not the case in initial experiments.

When plants containing Ph/P-Lox2-GUS or Cg/P-Lox2-GUS were crossed with several different plants containing the 35S-cre gene (not 35Sc-cre, see "Preparation of Transgenic Plants"), two different patterns of GUS expression were observed in the F1 seed (Fig. 3, C, Cg/P-cre embryos, no GUS staining). The 35S-cre plants were from lines analyzed in previous experiments in which the transformation selection marker, bounded by lox sites, was excised from the tobacco genome in cre by lox F1 progeny (Russell et al., 1992). Line Cre3 was the best at directing recombination in these previous experiments, and line Cre2 was the least effective. Crosses of lox2-GUS plants (both Ph/P-Lox2-GUS and Cg/P-Lox2-GUS plants used) with two plants from line Cre3 produced an activation pattern in F1 seed similar to that described above for Ph/P-cre and Cg/P-cre activation (Fig. 3A): a delay of about 1 d following initial expression of phaseolin and conglycinin promoters (Fig. 3E, and earlier stages shown in Fig. 5B at higher magnification). This result indicates that the GUS gene was not preactivated. In crosses of lox2-GUS plants with plants from line Cre2, F1 seed showed a pattern of GUS expression that indicated even later excision of the lox-bounded blocking fragment. No GUS activity was observed until embryos reached a late torpedo stage. Spots of GUS-expressing cells were observed first in the cotyledon-axis

Figure 5. Comparison of GUS gene activation by Cre expressed from different cre genes. These F1 embryos are shown at 1.6X the magnification of those in Figures 2 and 3. A, Embryos inheriting Cg/P-Lox2-GUS and 35Sc-cre dissected at 10 dap. B, Embryos inheriting Cg/P-Lox2-GUS and 35Sc-cre dissected at 10 dap. C, Embryos inheriting Cg/P-Lox2-GUS and 35Sc-cre dissected at 11 dap.
juncture region, and then in the cotyledons. In older embryos there were large patches as well as smaller spots of GUS-expressing cells. Endosperm from maturing seed also had localized spots of GUS activity. Surprisingly, one plant from the Cre3 line caused this same delayed and limited pattern of GUS activation (Fig. 3, C and D). This plant appeared to have reduced Cre expression. Its Cre3 siblings activated GUS expression no earlier than when Cre was regulated by the conglycinin and phaseolin promoters.

The gene construction that includes the 35S promoter/Cab leader and cre coding region, 35SC-cre, did direct the earlier activation of the GUS gene that was originally predicted. In F1 embryos from crosses of one Cg/P-Lox2-GUS plant with two independent 35SC-cre transformants, GUS was expressed in embryos entering the torpedo stage in all but the tips of the cotyledons and bottom of the axis. Shown in Figure 5A, these embryos are at higher magnification, but are of similar stages as some in Figure 2, A and C. The tip of the root axis did show GUS activity, following the expression pattern specific for the 35S promoter. Young torpedo-stage embryos stained entirely blue. This mimics the expression pattern for Cg/P-GUS (no activation required), with the addition of the blue axis tip. Thus, the blocked GUS gene appears to have been preactivated by Cre present in heart-stage embryos, as predicted from the expression pattern of this same promoter visualized using the GUS marker, 35SC-GUS, described above.

From the patterns of GUS gene activation, we infer that the initial 35S-cre construction directed lower and lower levels of expression than 35SC-cre, which probably expressed in a manner similar to 35SC-GUS. The better expression of the 35SC-cre gene, as compared to the 35S-cre gene, may be attributed to the different 5' untranslated leader sequences. In particular, the sequence surrounding the translation initiation ATG of the 35SC-cre gene is more similar to the consensus sequence desired for maximal translatability (Fig. 6; Kozak, 1986). It is not known if translational efficiency accounts for the difference in expression levels, or whether increased mRNA stability is possibly conferred by the Cab leader as well as other unknown factors.

**Nuclear Targeting of Cre**

The efficiency of Cre-mediated recombination could potentially be increased by targeting Cre protein to the nucleus.

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TTAATGTCC  crel-cre
AGCAATGSCCT  consensus
AACCATGGCC  Cabl-cre
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**Figure 6.** Comparison of sequences surrounding the translation initiation ATG in two different cre genes expressed from the 35S promoter. The top sequence is from the 35SC-cre gene, which has the natural sequence 5' to cre in the P1 genome upstream of the ATG (crel-cre). The bottom sequence is from the 35SC-cre gene, which has the leader from the Cab gene 22T (Cabl-cre). The middle sequence is the Kozak consensus for maximal translation, with the most important nucleotides underlined. Vertical dashes indicate the identities between the sequences surrounding the ATGs.

Cre is a small (38 kD) prokaryotic protein that obviously is able to enter the eukaryotic nucleus. However, by facilitating entry into the nucleus, the delay in timing between Cre expression and lox gene activation might be decreased. This hypothesis was tested by placing the SV40 large-T antigen nuclear-localization signal sequence at the N terminus of Cre expressed from an NTcre gene (see 'Materials and Methods'). Three independent Cg/P-NTcre transformants were crossed to a Cg/P-lox2-GUS line, and GUS expression was followed in F1 embryos to determine whether activation was more rapid than in embryos inheriting Cg/P-cre. The same 1-d delay in GUS expression was observed with both cre genes (Fig. 3A, and Fig. 5C at higher magnification). Thus, the addition of this targeting signal did not increase the in vivo efficiency of the Cre/lox recombination system.

**Lox2-GUS Is Preactivated in F2 Seed**

To determine whether the blocked GUS gene was activated in genetically effective cells in the F1 generation, plants were grown from F1 seed derived from crosses between Ph/P-lox2-GUS plants and Ph/P-cre or 35S-cre plants. GUS expression was observed in developing F2 seed produced by selfing each F1 plant. Preactivation of the GUS gene in the F1 generation, prior to F2 seed formation, can be determined in two ways. The GUS expression pattern in F2 embryos should not be delayed, as it is in the F1 embryos, if preactivation occurred. Also, genetic segregation predicts different ratios of GUS-expressing embryos for preactivation versus activation in the F2 seed.

Each of the original cre parents crossed to produce F1 seed was homozygous for the cre gene at a single locus. Each of the original lox parents was hemizygous (primary transformants) at a single locus for the interrupted GUS (lox2-GUS) gene. Therefore, each cross produces 50% of seeds inheriting both cre and lox, and 50% of seeds inheriting only cre. Seedlings that inherited both cre and lox were selected by resistance to kanamycin, the marker linked to the lox2-GUS gene. Each of these F1 plants is heterozygous for both cre and lox. Selfing of the F1 plant produces F2 seed in the following ratios of genotypes: 9 lox + cre:3 lox alone:3 cre alone:1 neither lox nor cre. If preactivation occurred in the F1 generation, cre is not necessary for activation in F2 seed, so all F2 seed inheriting lox would express GUS. A ratio of 3 blue:1 white embryos is expected in this situation. If activation is necessary for GUS expression, then cre must be inherited along with lox2-GUS, so a ratio of 9 blue:7 white embryos is expected. Ratios of 3 blue:1 white were observed in the F2 embryos of each selfed cre-lox F1 plant in samples of over 40 (for example, 31 blue embryos:12 white embryos). Thus, the genetic analysis indicates that the lox blocking fragment is removed in genetically effective cells prior to F2 seed development.

Results of the second assay for preactivation were consistent with the genetic data in that the timing of GUS expression in F2 embryos was not delayed. Similar expression patterns were observed in F2 embryos of two individual F1 plants derived from each of the following crosses: two independent Ph/P-lox2-GUS lines with one Ph/P-cre line and one Ph/P-lox2-GUS line with two 35S-cre lines. Spots of GUS activity

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appeared first in heart-stage embryos (not shown) and a thick blue ring was seen in mature hearts (Fig. 3F), clearly earlier than seen in the F1 embryos. The expression level appeared to be slightly reduced, since in young torpedo-stage embryos the axis stained a lighter blue and the axis tip remained white, even after the overnight incubation in GUS assay buffer. Reasons for this reduced expression are unknown.

**DISCUSSION**

To characterize the timing of Cre-mediated gene activation in seed, it was first necessary to establish the expression patterns of the two seed-specific promoters used in our system. By staining intact embryos we were able to visualize the timing and pattern of expression of the marker GUS gene directed by the promoter of either the French bean \( \beta \)-phaseolin gene or the soybean \( \alpha' \) subunit of the \( \beta \)-conglycinin gene. In each case the expression was earlier in the development of transgenic tobacco embryos than expected from previous reports. Soybean \( \beta \)-conglycinin genes (\( \alpha' \), \( \alpha \), and \( \beta \) subunits) are highly expressed in soybean embryos in mid- to late-maturation stages (Meinke et al., 1981; Walling et al., 1986). In situ hybridization analysis of \( \beta \)-conglycinin expression (both \( \alpha' \) and \( \beta \) subunit mRNAs hybridized to the probe) showed no expression in globular-, heart-, or cotyledon-stage soybean embryos (Perez-Grau and Goldberg, 1989). Mid- to late-maturation-stage embryos expressed \( \beta \)-conglycinin in both cotyledons and the axis. In this analysis, there also was no expression in the endosperm.

Although seed development in tobacco and petunia is much more rapid than in soybean, previous results indicated that transgenic tobacco and petunia plants expressed the \( \alpha' \) \( \beta \)-conglycinin promoter in the corresponding mid to late stages of embryo development (Beachy et al., 1985; Chen et al., 1988; Naito et al., 1988; Chamberland et al., 1992). Since promoter sequences to −257 bp upstream of the transcription start site were shown to be sufficient for temporal control, as well as for maximal expression (Chen et al., 1986, 1988), our 550-bp promoter fragment was sufficient to contain all of the regulatory sequences. Using GUS staining of individual embryos, we observed expression in heart-stage embryos. Since the same heart-stage expression was observed in embryos of many independent transformants, this pattern is not related to a position effect. The staining of isolated embryos, which allows the visualization of limited localized expression, may allow detection of expression that is not detected above background in analyses of protein or total RNA prepared from bulk seed samples, the assays used in the other studies. Therefore, the early timing and endosperm expression that we observe, which differ from the endogenous gene expression in soybean seed as determined by in situ hybridization, may result from expression of the \( \alpha' \beta \)-conglycinin promoter in a heterologous host or from a more sensitive assay. The expression we see in the cotyledons and axis of older embryos is in agreement with the expression pattern in maturation-stage soybean embryos. Chamberland et al. (1992) also observed uniform staining of the cotyledons and axis of mature embryos bearing a GUS gene expressed from the \( \alpha' \beta \)-conglycinin promoter. They did not determine GUS localization in earlier-stage embryos. They describe a light staining of the endosperm in mature seed, which indicates lower expression than we observe in that tissue.

The promoter of the French bean \( \beta \)-phaseolin gene has also been reported to be expressed in mid- to late-maturation stages (Sun et al., 1978; Murray and Kennard, 1984). In transgenic tobacco seeds analyzed as bulk samples, phaseolin protein (Sengupta-Gopalan et al., 1985) and \( \beta \)-phaseolin promoter-expressed mRNA (Riggs et al., 1989) or marker gene activity (Bustos et al., 1989) were first detected at a mid-maturation stage. Deletion analysis of this promoter in transgenic tobacco revealed a complex set of regulatory regions within the sequence between +20 and −795 (Bustos et al., 1991). Since our promoter fragment extends only to −410, it retains only a subset of these domains. It does include upstream activating sequence 1 and lack negative regulatory sequence 2, a combination that results in expression 3 d earlier than that directed by the full-length promoter region. Thus, the shorter length of our \( \beta \)-phaseolin promoter may explain the early expression that we observe in heart-stage embryos. However, we do not lose expression in the hypocotyl region of mature embryos, as was reported by Bustos et al. (1991) for a −418 promoter. In their report, longer promoters were expressed throughout the embryo except in the root meristem and cap. All promoter fragments analyzed directed expression in the endosperm as well, in agreement with our observations of endosperm expression.

The pattern of expression directed by the \( \alpha' \beta \)-conglycinin and \( \beta \)-phaseolin promoters in tobacco seed was very consistent among independent transformants in this study. Others have also observed a relative insensitivity to position effects of seed-specific promoters (Chen et al., 1986). Therefore, we assume that the pattern of Cre expression directed by the \( \alpha' \beta \)-conglycinin and \( \beta \)-phaseolin promoters is similar to the pattern of GUS expression from the same promoters, initiating at a heart stage and extending throughout the embryo in a young torpedo stage. Cre expressed in this manner is able to activate the \( \text{lox}^2 \)-GUS gene throughout the entire embryo and endosperm. The pattern of activation in the embryo follows the pattern of promoter activity. Spots of activated GUS activity are first detected at the junction between the cotyledons and embryo axis, the site of highest seed promoter expression. A direct correlation between amount of Cre protein and frequency of recombination was previously established (Sauer and Henderson, 1988). Blue spots are first seen in torpedo-stage embryos, about 1 d in development after the \( \alpha' \beta \)-conglycinin and \( \beta \)-phaseolin promoters are first active, at the heart stage. This then is the period of time required for Cre protein to be synthesized, enter the nucleus, identify \( \text{lox} \) sites within the plant genome, and recombine these sites, producing a functional gene.

Entering the nucleus does not seem to be a limiting factor, since the addition of a nuclear-targeting signal to Cre did not improve the timing of \( \text{lox}^2 \)-GUS activation. Cre is a small, 38-kD protein that should be able to pass through the nuclear pore complex (Garcia-Bustos et al., 1991). However, small nuclear-localized proteins do contain localization signals to facilitate their entry (Raikhel, 1992). The SV40 large T-antigen nuclear localization signal did effectively direct the localization of T7 polymerase to the plant cell nucleus (Lass-
Comparison of the efficiencies of recombination directed by our 35SC-cre and 35S-cre genes supports the importance of the mRNA sequence in contributing to the level of protein expression. Inferring from the timing of lox2-GUS gene activation, the 35SC-cre gene is expressed much more highly than the 35S-cre gene. These genes do have different 3' ends: Ocs 3' versus Nos 3', which could contribute to mRNA processing and stability. However, there is a striking difference in the sequence surrounding the translation-initiating ATG, 35SC-cre having an almost perfect consensus sequence (Kozak, 1986) and 35S-cre having little homology. Thus, mRNA translatability may be the predominant basis for the discrepancy in Cre expression.

During these experiments we also saw differences in levels of Cre expression, inferred from the timing of lox2-GUS gene activation, among plants belonging to the same 35S-cre line. Several different 35S-cre plants from the Cre3 line were used in crosses to activate lox2-GUS. They were all grown from seed produced by selfing an R1 plant that was homozygous at a single locus for the linked selection marker. Thus, these plants were expected to be genetically identical. Two of them directed the same pattern of lox2-GUS-activated GUS activity in F1 seed as expected, whereas one directed much less activation. Reduced Cre activity in this plant, as compared to its siblings, could be due to methylation of the 35S-cre transgene. Variable loss of transgene expression among siblings has been correlated with methylation in some studies (Lin et al., 1990; Kilby et al., 1992; Asaad et al., 1993). We have not compared the methylation of the 35S-cre gene in this plant and its siblings.

The reduced recombination directed by this 35S-cre plant, and also by the plants of the Cre2 line, produces a spotting pattern on maturing cotyledon-stage embryos. Small spots indicate sites of recent lox2-GUS activation, whereas larger spots indicate an earlier recombination event and include cells descending from the one in which lox2-GUS was activated. Limited recombination provides a system in which cell lineages may be followed. Limited recombination could be directed by low expression of Cre in any tissue, producing a mosaic pattern of expression of a target gene. This system could be used to restore function of a wild-type gene in the corresponding mutant background, in a cell-localized manner. Effects of cell-autonomous mutant restoration on the tissue would indicate whether a cell's response to the gene product is transmitted among the cells of the tissue or remains limited to the cell in which it is expressed. This study on tissue-specific Cre-mediated activation of a GUS marker gene, whose expression is initially blocked by a lox-bounded fragment, provides a basis for applying the Cre/lox recombination system to these types of developmental studies.

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