Identification of an Enhancer Element for the Endosperm-Specific Expression of High Molecular Weight Glutenin

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Genes encoding high molecular weight (HMW) glutenin, a wheat seed storage protein, are expressed only in the developing endosperm. It was previously demonstrated that sequences essential for endosperm-specific transcription reside within 436 base pairs upstream of the initiation codon for HMW glutenin translation. We have further analyzed this region by testing the ability of a series of truncated HMW glutenin promoter fragments to enhance transcription from an adjacent heterologous promoter. The activity of these hybrid promoters was determined by measuring the expression of a linked β-glucuronidase (GUS) reporter gene in transgenic tobacco plants. An HMW glutenin promoter fragment spanning nucleotides −375 to −45 relative to the transcription start site was found to stimulate GUS expression in tobacco seeds when inserted in either orientation upstream of the heterologous promoter. Furthermore, this fragment could also potentiate transcription when located 3′ to the GUS reporter gene. Stimulation of GUS gene expression in transgenic tobacco seeds did not occur until 9 days to 12 days after anthesis, coincident with the onset of storage protein synthesis in the developing tobacco and wheat seed, and was confined to the endosperm tissue. By testing progressively shorter promoter fragments, the enhancer element responsible for this pattern of expression was localized to a 40-base pair region some 170 base pairs upstream of the start site for HMW glutenin transcription.

INTRODUCTION

Storage proteins accumulate in significant quantities during seed development and serve as a source of reduced nitrogen for early stages of seedling growth. The developing wheat grain consists predominantly of endosperm tissue, which is the site of synthesis and deposition of prolamin storage proteins. The high molecular weight (HMW) subunits of glutenin are a group of prolamins of 70 kD to 90 kD and constitute approximately 10% of the total storage protein of wheat grain (Kasarda et al., 1976). Modern wheat varieties each contain between three and five different HMW glutenin subunits, and these proteins play a major role in determining the viscoelasticity and, hence, the bread-making quality of wheat flour (Payne et al., 1984).

Synthesis of seed storage proteins is subject to developmental, organ-specific, and environmental controls. Evidence suggests that the coordinate expression of cereal storage protein genes is controlled primarily at the level of transcription (Soave and Salamini, 1984; Bartels and Thompson, 1986; Heidecker and Messing, 1986; Kreis et al., 1986). A deletion analysis of the promoter for the HMW glutenin gene Glu-D1-2, which encodes subunit 12, has recently established that sequences necessary for endosperm-specific transcription in transgenic tobacco plants reside within 436 bp upstream from the translation initiation codon (Colot et al., 1987; Robert et al., 1989). Here we describe further experiments that have enabled us to localize these cis-acting sequences.

RESULTS

Vector Construction and Analysis of Transformed Tobacco Plants

The plasmid vector pBI121.5 that was used for the gene fusion experiments described in this paper, as shown in Figure 1, contains the reporter gene encoding β-glucuronidase (GUS) (Jefferson et al., 1986, 1987) under the tran-
The acceptor plasmid for all the glutenin promoter fragments tested was pBI121.5, which contains a truncated CaMV 35S RNA promoter, the GUS reporter gene, and the 3' end of the nos gene cloned into the polylinker region of pBin19 (see text). The coordinates on the HMW glutenin promoter fragments denote the distance in base pairs of the end points from the initiation site for HMW glutenin transcription. Arrows indicate the polarity of the promoter fragments.

**Figure 1.** Plasmid Constructs Used for HMW Glutenin Promoter Analysis.

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It has been shown that sequences necessary for endosperm-specific transcription of the Glu-D1-2 gene reside on a 429-bp DNA fragment spanning nucleotides −436 to −8 from the translation initiation codon (Colot et al., 1987; Robert et al., 1989). This corresponds to nucleotides −375 to +54 relative to the transcription start site assuming a transcription initiation site identical to that determined for the related Glu-D1-1 gene (Sugiyama et al., 1985). A consensus TATA box sequence also resides on this fragment in the expected position, as shown in Figure 2, 30 bp upstream of the proposed transcription initiation site (Thompson et al., 1985). Although the TATA box is necessary for correct initiation of transcription by RNA polymerase II (Kovacs and Butterworth, 1986), other cis-acting sequences, usually located upstream from the TATA box, are known to be involved in the transcriptional regulation of a number of genes (for reviews, see Maniatis et al., 1987; Guarente, 1988; Jeang and Khoury, 1988). Therefore, it seemed likely that control elements necessary for regulated expression of the HMW glutenin gene may be located between the TATA box and nucleotide −375 (relative to the transcription initiation site). Accordingly, a promoter fragment spanning nucleotides −375 to −45 was inserted in the normal orientation upstream of the truncated CaMV promoter of pBI121.5 generating pGUS45 (Figure 1). GUS specific activity was then measured in tissue extracts of transgenic tobacco plants containing pBI121.5 and pGUS45. Figure 3 shows that the levels of GUS activity found in 30 DAA seeds derived from pGUS45 transformants varied considerably between plants. Such variation in expression of transferred genes in plants is well documented (Jones et al., 1985; Nagy et al., 1985; Dean et al., 1988) and could be due to rearranged sequences, methylation, so-called "position effects," or physiological variation during seed development. The wide variation in GUS activity among individual plants transformed with the same plasmid clearly justified our decision to analyze a large number of independent transformants. Despite this variation, it is apparent that all except three pGUS45 transformants exhibited seed GUS levels significantly above any of the pBI121.5 transformants (Figure 3). Table 1 shows that seed extracts from plants harboring pGUS45 were found to contain on average eightfold to ninefold higher levels of GUS activity than the pBI121.5 transformants. However, the presence of this glutenin DNA
Figure 2. Nucleotide Sequence of the Glu-D1-2 Promoter Region from the Allele Encoding Subunit 12 Showing Deletion End Points Used To Construct the Hybrid Promoters.

The TATA box element is boxed and the presumed transcription initiation site is indicated by a horizontal arrow. Potential regulatory sequence motifs referred to in the text are also indicated. The Glu-D1-2 promoter for the allele encoding subunit 12 of which pGUS148, pGUS186, and pGUS234 were derived differs at only two nucleotide positions within the region shown (C instead of T at -70, and A instead of G at -114, A. Goldsbrough, L. Schnick, L. Robert, and R. Flavell, manuscript submitted) and, therefore, does not affect the interpretation of the results.

fragment upstream of the CaMV promoter was found not to affect GUS gene expression in leaves of transgenic plants (Table 1). Therefore, sequences are present on this DNA fragment that can stimulate transcription from a heterologous promoter in an organ-specific manner.

To determine whether or not the function of this control element is dependent upon its position relative to the TATA box, the fragment was inserted upstream of the CaMV promoter in the reverse orientation (pGUS45R), and downstream from the GUS reporter gene (pGUS45D) (Figure 1). The GUS activities measured in seed extracts from plants transformed with pGUS45R were very similar to those found in pGUS45 transformants (Figure 3, Table 1). When the fragment was located 3' to the GUS gene, however, the levels of GUS activity were much lower, but approximately one-quarter of the plants had levels higher than the highest level in the control plants (Figure 3, Table 1). Again, there was no significant stimulation of GUS transcription from the CaMV promoter in the leaves of pGUS45R and pGUS45D transformants (Table 1). Therefore, the promoter element carried on this 330-bp fragment possesses enhancer-like properties in that it can function in both orientations and is able to exert an effect over a relatively large distance (approximately 2 kb). Insertion of two copies of this fragment upstream of the CaMV promoter (pGUS45T) did not result in any significant increase in seed (or leaf) GUS activity relative to the levels found in pGUS45 transformants. However, we note that one of the pGUS45T transformants expressed the highest levels of seed GUS activity measured among the total transformants used for this analysis (Figure 3, Table 1).

Glutenin Enhancer Directs both Spatial and Temporal Programs of Gene Expression

The HMW glutenin promoter directs transcription of a linked CAT reporter gene in only (or predominantly only) the endosperm of transgenic tobacco seeds (Robert et al., 1989). Therefore, seeds from tobacco plants transformed...
Table 1. GUS Activity in Seeds and Leaves of Transgenic Tobacco Plants

<table>
<thead>
<tr>
<th>Construct</th>
<th>GUS Specific Activitya</th>
<th>Endosperm-Specific Expression</th>
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<tbody>
<tr>
<td></td>
<td>Seedsb</td>
<td>Leaves</td>
</tr>
<tr>
<td>pBI121.5</td>
<td>0.32 ± 0.12 (7)</td>
<td>0.10 ± 0.02 (3)</td>
</tr>
<tr>
<td>pGUS45</td>
<td>2.74 ± 1.59 (14)</td>
<td>0.10 ± 0.12 (6)</td>
</tr>
<tr>
<td>pGUS148</td>
<td>3.56 ± 2.25 (14)</td>
<td>0.15 ± 0.05 (10)</td>
</tr>
<tr>
<td>pGUS163</td>
<td>1.51 ± 1.00 (10)</td>
<td>0.12 ± 0.10 (6)</td>
</tr>
<tr>
<td>pGUS186</td>
<td>0.51 ± 0.31 (15)</td>
<td>0.19 ± 0.15 (12)</td>
</tr>
<tr>
<td>pGUS234</td>
<td>0.26 ± 0.22 (18)</td>
<td>0.12 ± 0.10 (5)</td>
</tr>
<tr>
<td>pGUS283</td>
<td>0.34 ± 0.16 (9)</td>
<td>0.13 ± 0.07 (9)</td>
</tr>
<tr>
<td>pGUS45R</td>
<td>2.36 ± 1.76 (10)</td>
<td>0.13 ± 0.09 (9)</td>
</tr>
<tr>
<td>pGUS45D</td>
<td>0.66 ± 0.58 (9)</td>
<td>0.13 ± 0.07 (7)</td>
</tr>
<tr>
<td>pGUS45T</td>
<td>3.35 ± 2.05 (13)</td>
<td></td>
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a GUS activities are expressed as picomoles of 4-methylumbelliferone per minute per microgram of protein and represent the mean and standard errors for plants expressing GUS at higher than background (untransformed) levels (0.06 and 0.00 pmol of 4-methylumbelliferone/min/μg of protein for seed and leaf, respectively). All plants were grown at the same time in the glasshouse and tissue samples taken and assayed together. Values in parentheses are the number of plants used in the calculations of means and standard errors. - denotes leaf assays done in only two plants; therefore, means and standard deviation not calculated.

b Seeds were harvested at 30 DAA. Seed GUS activities of individual plants are shown in Figure 3.

with our hybrid promoter-GUS fusions were analyzed histochemically to identify the site of GUS expression. Histochromal staining, shown in Figure 4A, revealed that GUS activity was restricted to the endosperm tissue of seeds from plants containing pGUS45, pGUS45R, pGUS45D, and pGUS45T (Figure 4A, Table 1). Prolonged incubation (overnight) did not result in staining of the embryo tissue. This pattern of expression was not observed for pBI121.5-transformed seeds. On the contrary, GUS activity, but a lower level, was localized to discrete parts of the embryo, usually the radicle end, as shown in Figure 4B. Occasionally, staining of isolated clusters of endosperm cells was also observed.

During wheat endosperm development, HMW glutenin mRNAs are first detected around 10 DAA and the amounts increase until the mid-developmental phase (about 20 DAA), after which time the levels decline (Bartels and Thompson, 1986). To determine whether this element directs an equivalent temporal program of GUS expression in transgenic tobacco, GUS activity was measured in seeds harvested at various stages of development. For plants transformed with pGUS45, pGUS45R, pGUS45D, and pGUS45T, stimulation of GUS synthesis was first detected between 9 DAA and 12 DAA, coincident with the time of appearance of glutenin mRNA in developing wheat grain. From Figure 5, it can be seen that between 15 DAA and 18 DAA a rapid accumulation of GUS activity occurred, reaching a maximum at 21 DAA, after which time the activity diminished. This profile mimics the normal time course of glutenin gene expression in the developing wheat grain. Seeds from plants transformed with pBI121.5, however, showed only a slight increase in GUS activity during the middle stages of seed development, which again declined during the latter stages of seed maturation (Figure 5). Therefore, the enhancer sequences present on the 330-bp promoter fragment are able to specify both the spatial and temporal programs of HMW glutenin gene transcription in transgenic tobacco.

Delineation of Enhancer Element by Deletion Analysis

To determine the minimum amount of upstream glutenin DNA required for regulated expression, we tested successively shorter fragments for their ability to modulate transcription from the Δ90 CaMV promoter. These fragments extend from the HindIII site at −375 toward the TATA box and terminate at various points upstream of −45. Insertion of these fragments upstream of the CaMV promoter of pBI121.5 (in the normal orientation relative to the TATA box) gave rise to pGUS148, pGUS163, pGUS186, pGUS234, and pGUS283 (Figures 1 and 2). Analysis of tobacco plants transformed with these plasmids showed that removal of 103 bp from the 3' end of the 330-bp promoter fragment (pGUS148) caused a marked reduction of seed GUS activity relative to pGUS148. Nevertheless, this still represents an approximate fivefold stimulation of pBI121.5 CaMV promoter activity in seeds (Figure 3, Table 1). A further decrease in CaMV promoter activity resulted from deletion of an extra 23 bp from the glutenin upstream sequences (pGUS186), giving rise to levels of seed GUS activity that were similar
Figure 4. Localization of Seed GUS Activity in Tobacco Plants Transformed with pGUS45 and pBI121.5.

Seeds harvested at 21 DAA were sectioned and analyzed histochemically for GUS activity as described in Methods.

(A) Cross-section of seed from a typical pGUS45-transformed tobacco plant. GUS activity was clearly visible in the endosperm after 1 hr to 2 hr incubation in X-gluc. Staining of the embryo was not observed even after prolonged incubation.

(B) Section of seed from a typical pBI121.5-transformant. Incubation in X-gluc was for 6 hr, at which time staining of the embryo was clearly visible. Staining of isolated clusters of endosperm cells can also be seen. En, endosperm; Em, embryo.

Figure 5. Expression of GUS Activity in Developing Seeds from Individual Tobacco Plants Transformed with pGUS Plasmids.

Seeds from an untransformed plant (●) and transformants containing pBI121.5 (○), pGUS45 (▲), and pGUS45T (△) were collected at different time points after anthesis. GUS activity was assayed on approximately 100 seeds at each time point and normalized to the protein content of the sample. MU, 4-methylumbelliferone.

Discussion

It has been shown that a 436-bp HMW glutenin promoter fragment containing the entire untranslated leader se-
quence and 375 bp of DNA upstream from the transcription start site is sufficient for endosperm-specific transcription in transgenic tobacco (Colot et al., 1987; Robert et al., 1989). The results of experiments described here have enabled us to characterize further the elements necessary for the tissue-specific expression of HMW glutenins. By measuring the activity of various hybrid promoter-GUS fusions in transgenic tobacco plants, we were able to show that sequences required for directing endosperm-specific expression reside within 345 bp upstream of the TATA box. Furthermore, we demonstrated that these sequences can functionally interact with a heterologous promoter in an orientation-independent and position-independent manner. These are characteristics of enhancer elements (Serfling et al., 1985; Jeang and Khoury, 1988) which, unlike upstream activation sites or upstream promoter elements, can function when placed downstream of the transcription start site (for reviews, see Maniatis et al., 1987; Stanway et al., 1987; Guarante, 1988). A number of cis-acting control elements have been identified that can function in either orientation upstream of plant genes (Flurk et al., 1986; Nagy et al., 1987; Dean et al., 1989). Some of these elements, however, do not activate transcription when located downstream from the promoter (Timko et al., 1985). Apart from the CaMV 35S RNA enhancer (Nagy et al., 1987; Fang et al., 1989), only one other plant promoter element (the β-conglycinin enhancer) has been reported to activate transcription when placed 3' to the transcription start site (Chen et al., 1988).

Interestingly, GUS expression specified by the 345-bp promoter fragment was confined to the endosperm tissue of transgenic tobacco plants as in wheat despite the fact that tobacco seed storage proteins accumulate in both endosperm and embryo (Sano and Kawashima, 1983; Kuhlemeier et al., 1987). Furthermore, the time of appearance of GUS activity was similar to that of both glutenin synthesis in wheat and storage protein synthesis in tobacco (Erdelska, 1985; Bartels and Thompson, 1986; Kuhlemeier et al., 1987) in accordance with previous observations regarding the behavior of the HMW glutenin promoter in transgenic tobacco (Robert et al., 1989). There was a small but reproducible increase in GUS activity observed during development of pBI121.5-transformed seeds, presumably because of the GUS activity in the embryo shown in Figure 4B. A similar pattern of expression with this promoter has been shown by Benfey et al. (1989).

In all plants studied, GUS activity was seen to decline in seeds after 21 DAA. During wheat and tobacco seed development, storage protein synthesis decreases at the onset of seed drying and mRNA levels decline considerably (Bartels and Thompson, 1986). It is reasonable, therefore, to assume that the decrease in accumulation of GUS levels at around 24 DAA is due to similar controls over protein synthesis. However, the GUS levels were not maintained at the mid-developmental peak value but decreased during seed maturation, suggesting that the GUS enzyme is unstable during seed drying in contrast to the behavior of chloramphenicol acetyltransferase, which remains at an elevated level throughout the latter stages of seed development in transgenic tobacco (Colot et al., 1987; Robert et al., 1989).

It should be noted that the relative rankings of the seed GUS activities among individual transformants remained constant irrespective of the developmental stage, as exemplified in Figure 5. This result has allowed us to make meaningful comparisons between GUS activities from different plants at the 30 DAA stage chosen (Figure 3 and Table 1).

By testing successively smaller promoter fragments for their ability to confer endosperm-specific enhancement on the δ90 CaMV promoter, we were able to define the 3' limits of the glutenin enhancer sequences. The decreased potency of the δ163 promoter fragment relative to the −148 deletion suggests that the 3' boundary of the control element resides between −148 and −163 nucleotides upstream from the transcription start site. However, it should be emphasized that the δ163 promoter fragment still retains the ability to enhance transcription in a tissue-specific manner. Therefore, it might be argued that the decreased enhancement is due to a more indirect effect such as reduced accessibility of a binding site to a trans-acting factor or an unfavorable alignment of the enhancer sequences with respect to the CaMV TATA box. Complete elimination of endosperm-specific regulation does occur on deletion to position −186. Perusal of the −148 to −186 interval reveals several DNA sequence motifs that may be relevant to HMW glutenin gene expression. These include a direct repeat of the pentanucleotide sequence TTGCT, a pair of GCTCC direct repeats, and a sequence possessing imperfect dyad symmetry. None of these sequences appears anywhere else in the HMW glutenin promoter region (Figure 2). Regarding the possible involvement of the direct repeats in the binding of transcription factors, it may be significant that one of each pair of these motifs is disrupted by the −163 deletion, resulting in a decrease in GUS expression in the endosperm.

In the presence of the wheat promoter DNA, the activity of the δ90 CaMV promoter in the embryo appeared to be suppressed. The suppression was relieved to the −186 and −234 deletions, implying that the element that produces endosperm activity is also responsible for the suppression of expression in the embryo. When the CaMV −343 to −90 enhancer is added to the δ90 CaMV promoter, the embryo expression is not reduced but gene expression is extended to the cotyledon end of the embryo also (Benfey et al., 1989). Therefore, the silencing effect of the wheat glutenin enhancer is not seen with all enhancers.

Examination of the DNA sequence for a number of other cereal prolamin genes has revealed a conserved sequence of about 30 bp lying approximately 300 bp upstream of the translation initiation codon (Forde et al., 1985; Kreis et
genes of wheat (Kreis et al., 1986; Colot et al., 1989). As yet, a role has not been assigned to this sequence, although it is contained within a 160-bp region that is necessary for endosperm-specific expression of low molecular weight glutenin (Colot et al., 1987). The element has the consensus sequence TGACATGTAATAAGATGAGT and incorporates two smaller conserved motifs (underlined). One motif is identical to the consensus sequence TGCAAAAG is present in the important -148 to -186 interval of the HMW glutenin promoter region. Although a sequence exhibiting poor homology to the -300 element has been identified 450 bp upstream of a Glu-B1-2 gene (Halford et al., 1987), it lies outside of the region shown to be essential for the tissue-specific regulation of HMW glutenin gene expression (Colot et al., 1987; Robert et al., 1989). Nevertheless, a variant of the -300 element core sequence (TGCAAAAG) is present in the important -148 to -186 interval of the HMW glutenin promoter. However, this sequence is not disrupted by the -163 deletion and has homologs at -257 and -353 that remain unaffected by the “knockout” -186 deletion. Therefore, if this sequence does play a role in the regulation of HMW glutenin gene expression, it is likely to do so not in isolation, but in combination with other neighboring sequences such as the direct repeats. Indeed, it is becoming clear that many enhancer elements are composed of multiple short sequence motifs that bind combinations of transcription factors to confer inducibility, tissue specificity, or general enhancement on promoters (Pfeifer et al., 1987; Schirn et al., 1987; Jones et al., 1988; Schüle et al., 1988; von der Ahe et al., 1988; Dynan, 1989).

Alternatively, this motif may not be involved directly in the binding of regulatory proteins but has some other function in regulating HMW glutenin gene expression. In this respect, it is interesting that this sequence forms part of a more extensive A + T-rich tract that may be intrinsically bent (Koo et al., 1986; Koo and Crothers, 1988). Bent DNA has been identified in the regulatory regions of a number of prokaryote and eukaryote genes (Poljak and Gralla, 1987; Umek and Kowalski, 1987; Bauer et al., 1988; Inokuchi et al., 1988). Further experiments are in progress to determine the precise role of these sequences.

METHODS

Vector Construction

The plasmid vector pBl121.5 (Figure 1) was designed for the analysis of transcriptional regulatory signals and its construction will be described in detail elsewhere (R. Jefferson, A. Goldsbrough, and M. Bevan, manuscript in preparation). It is a derivative of the binary vector pBin19 (Bevan, 1984) and carries the Escherichia coli uidA reporter gene encoding GUS (Jefferson et al., 1985, 1987) under the transcriptional control of a truncated CaMV 35S RNA promoter. A polylinker sequence is located 90 bp upstream from the transcription start site and a transcription terminator is provided by the polyadenylation signal of the nopaline synthase gene (nos) downstream from the GUS gene.

Construction of HMW Glutenin Promoter-GUS Fusions

DNA fragments carrying 3′ deletions of the HMW glutenin promoter were obtained by BAL-31 exonuclease or exonuclease III/S1 nuclease digestion of the two cloned Glu-D1-2 genes specifying subunits 10 and 12, respectively, before DNA sequence analysis (Thompson et al., 1985; A. Goldsbrough, D. Schnick, L. Robert, and R. Flavell, manuscript submitted). These DNA fragments were analyzed for the extent of 3′ deletion by polyacrylamide gel electrophoresis and finally by dideoxy sequencing in pUC9 or pUC19 using the universal primer (Sanger et al., 1977). The deletions originate within the HMW glutenin structural gene and extend through the TATA box, terminating 45, 148, 163, 186, 234, and 283 nucleotides upstream from the transcription initiation site (Figure 2). These fragments were excised from the pUC9 and pUC19 derivatives using the HindIII site located 375 nucleotides upstream of the transcription start site (Figure 2) and appropriate polylinker restriction sites adjacent to the deletion end points and subsequently inserted into the pBl121.5 polylinker. This gave rise to pGUS45, pGUS148, pGUS163, pGUS186, pGUS234, and pGUS283, respectively, in which the truncated HMW glutenin promoter fragments are located upstream of the CaMV promoter in the correct orientation relative to the TATA box (Figure 1). The largest of these promoter fragments (containing the -45 deletion) was also inserted in the reverse orientation, and in duplicate in the normal orientation upstream from the truncated CaMV promoter to generate pGUS45R and pGUS45T, respectively. pGUS45D was constructed by insertion of this fragment in the normal orientation downstream of the terminator for GUS transcription. All plasmid constructions are shown in Figure 1.

Plant Transformation

pBl121.5-derived plasmids were mobilized from E. coli strain MC1022 (Casadaban and Cohen, 1980) into the nononcogenic Agrobacterium tumefaciens strain LBA4404 (Hoekema et al.,
were eliminated from the analysis. EMBO J. 8, 2195-2202.

All of the regenerated plants were transferred to soil and grown to maturity at the same time in the same glasshouse. On flowering, 179 gave rise to rooted cuttings on kanamycin (200 pg/mL). From a total of 500 leaf discs, 375 shoots were formed with 100 pg/mL kanamycin sulfate and 500 pg/mL carbenicillin. Shoots were transferred to MS medium without hormones and containing a reduced concentration of carbenicillin (200 μg/mL). From a total of 500 leaf discs, 375 shoots were taken, of which 179 gave rise to rooted cuttings on kanamycin. All of the regenerated plants were transferred to soil and grown to maturity at the same time in the same glasshouse. On flowering they were self-pollinated. Two of these plants exhibited an unusual morphology and several others failed to produce seeds. These were eliminated from the analysis.

GUS Assay

GUS assays were performed essentially as described previously (Jefferson 1987; Jefferson et al., 1987). One hundred seeds or an individual leaf disc (diameter 10 mm) were grown in 1 mL of extraction buffer (Jefferson, 1987) and the resulting tissue extracts cleared by centrifugation at 4000 rpm for 15 min. Reactions were carried out at 37°C in a 500-μL volume containing 50 μL of plant extract and 450 μL of assay buffer (Jefferson, 1987). At several intervals after initiation of the reaction, 50-μL portions were removed and mixed in a microtitre plate with 150 μL of 0.2 M Na2CO3 to stop the reaction. Fluorescence was measured using a Titertek Multiskan II (Flow Laboratories, Ltd.) calibrated against 1 μM 4-methylumbelliferone. Protein concentrations of plant extracts were determined by the dye-binding method of Bradford (1976) with a kit supplied by Bio-Rad Laboratories. Absorbance at 600 nm was measured in microtitre plates using a Titertek Multiskan Plus spectrophotometer.

Histochemistry

GUS enzyme activity in transgenic tobacco seeds was localized by histochemical staining with X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) (Jefferson, 1987; Jefferson et al., 1987). Sections (55 μm) of 21-day-old seeds were made using a cryostat and transferred directly onto glass microscope slides. Staining was carried out by incubation in 50 mM NaH2PO4 (pH 7.0) containing 1 mg/mL X-gluc (Biosynth AG, 9422 Staad, Switzerland) at 37°C for 30 min to 12 hr. A fixation step was not included.

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