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Use of microprojectile bombardment in transient expression assays to analyze protochlorophyllide reductase gene expression in greening maize seedling leaf cells

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USE OF MICROPROJECTILE BOMBARDMENT IN TRANSIENT EXPRESSION ASSAYS TO ANALYZE PROTOCHLOROPHYLLIDE REDUCTASE GENE EXPRESSION IN GREENING MAIZE SEEDLING LEAF CELLS

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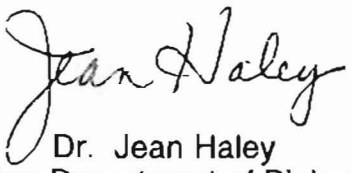
APPROVED BY:



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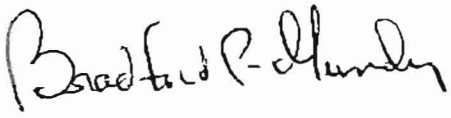

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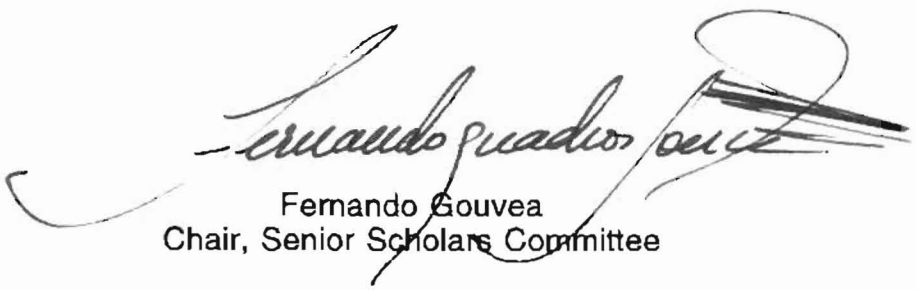

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Table of Contents

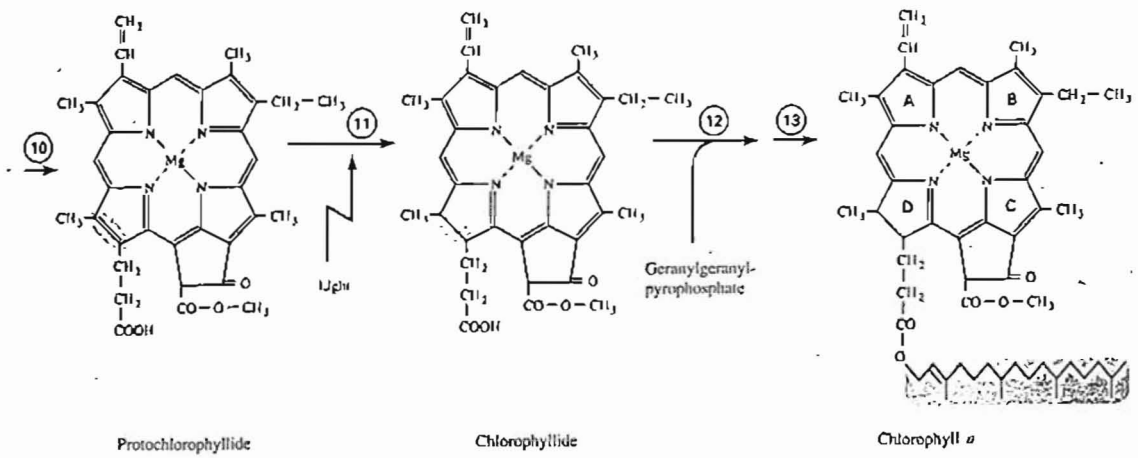
ABSTRACT

In young cells of leaf meristems the progenitors of chloroplasts are small organelles known as proplastids, which divide and differentiate into chloroplasts. However, in the absence of light, proplastids undergo a different sequence of development and become etioplasts. When light is supplied to etiolated plants during the "greening" process, etioplasts differentiate into chloroplasts containing chlorophyll. An important light-dependent step in chlorophyll biosynthesis is the photoreduction of protochlorophyllide to chlorophyllide by the NADPH:protochlorophyllide reductase (PCR) enzyme. This enzyme is present at high activity only in etiolated tissue and during early stages of light-induced chlorophyll synthesis. The enzyme and its corresponding mRNAs decrease dramatically with prolonged exposure to light. We have investigated the light-dependent transcriptional regulation of a PCR gene in greening maize leaf cells using a transient expression assay based on microprojectile bombardment. The promoter region was isolated and cloned into a β-glucuronidase (GUS) reporter gene expression plasmid. We have used this chimeric plasmid in tungsten particle bombardment of both etiolated and greening maize seedling leaves to determine whether the cloned promoter region contains regulatory sequences that control light-responsive PCR gene expression.

INTRODUCTION

Aside from giving plant leaves their green color, chlorophyll is a necessary pigment in photosynthesis. When a photon of light energy strikes a molecule of chlorophyll, an electron in that molecule is raised to a higher energy level. Energy is transferred by resonance to a reaction center chlorophyll molecule that subsequently donates a high-energy electron to a quinone in the electron transport chain. Energy conserved as electrons move through the transport chain is eventually used to fix carbon dioxide into organic compounds (Lehninger, 1992).

When grown in the dark, angiosperm seedlings do not accumulate chlorophyll. Their yellow color is due primarily to the presence of carotenoids. Dark grown seedlings do, however, accumulate significant amounts of the chlorophyll precursor protochlorophyllide *a*. Protochlorophyllide is converted to chlorophyllide by the reduction of the double bond between carbons 7 and 8 which is catalyzed by the enzyme NADPH: protochlorophyllide oxidoreductase (PCR):



Chlorophyllide is subsequently converted to chlorophyll by the addition of a phytol tail. In angiosperms the reduction of protochlorophyllide requires light, but in gymnosperms and most algae chlorophyll can be synthesized in the dark (Lehninger, 1992).

In very young cells of leaf meristems the progenitors of chloroplasts are small organelles known as proplastids which divide and differentiate into chloroplasts. In monocotyledons, such as maize, oat, barley, and wheat, the development of chloroplasts from proplastids is straight-forward when growth occurs in the light. However, in the absence of light, proplastids undergo a different sequence of development and become etioplasts. These plastids lack chlorophyll and do not contain extensive thylakoid membranes. When light is supplied to etiolated plants, etioplasts differentiate into chloroplasts through the process of "greening" (Anderson and Beardall, 1991). Tissues that are developing photosynthetic competence during the greening phase provide a useful system for studying the mechanisms of chlorophyll biosynthesis and other developmental events.

The light-dependent regulatory step in chlorophyll synthesis appears to be the formation of chlorophyllide. Some gymnosperms and algae synthesize an enzyme that reduces protochlorophyllide whether or not light is present, allowing these organisms to synthesize chlorophyll in the dark. The enzyme that catalyzes light-independent chlorophyll synthesis is expressed from a chloroplast-encoded gene. However, in angiosperms, including monocots such as maize and dicots such as peas, the conversion of protochlorophyllide to chlorophyllide is known to be a light-requiring process. The light-dependent form of PCR is encoded in the nuclear DNA, synthesized in the cytoplasm and subsequently imported into the chloroplast

with cleavage of an N-terminal transit peptide. The photoreduction of protochlorophyllide can be readily detected in leaf tissues of plants grown in the dark due to the marked difference in the spectroscopic properties between protochlorophyllide and chlorophyllide (Beer and Griffiths, 1981). Protochlorophyllide accumulation in etioplasts inhibits the synthesis of a precursor 5-aminolevulinate, but this inhibition is relieved by the photoreduction of protochlorophyllide to chlorophyllide by the PCR enzyme. At least one form of this enzyme is present at high activity only in etiolated tissue and during the early stages of light-induced chlorophyll synthesis. The levels of the PCR enzyme and its corresponding mRNA decrease 10-20 fold with prolonged exposure to light (Forreiter et al., 1990).

The effect of light on NADPH: protochlorophyllide oxidoreductase and its mRNA has been studied in several dicotyledonous and monocotyledonous plant species (Forreiter et al., 1990). Because PCR is believed to play an important role in the control of light-dependent chloroplast development in higher plants, it seems reasonable to conclude that the effect of light on the presence and activity of the enzyme would be similar in all higher plants that have etioplasts in the absence of light, that is, in all flowering plants. Experimental results support this hypothesis because, in all of these species, the illumination of etiolated seedlings leads to a rapid decline in both the activity and the content of the enzyme protein as well as the mRNA. However, the rate and the extent of the decline vary in different species, and these differences probably reflect different kinds of gene regulation. Studies concerning the light-regulated expression of the protochlorophyllide reductase gene in maize leaf cells were conducted by Earl Lewis at Colby College (personal communication) and some differences from published reports were found involving mRNA

accumulation. Specifically, the maize PCR mRNA disappears more rapidly than had previously been reported (Forreiter et al., 1990), showing about a 10-fold decrease in abundance within the first three hours of leaf exposure to light. In this respect, the maize gene appears to be regulated similarly to the genes of other monocots such as barley, and differently from the genes of the less light-sensitive dicots such as pea and *Arabidopsis*. The rapid decline in mRNA indicates that the PCR gene is negatively-regulated by light and also implies that control at the level of transcription is important.

In barley, the light-responsive down-regulation of PCR gene expression has been shown to be due in part to the negative regulation of gene transcription, under the control of the phytochrome photoreceptor (Mosinger et al., 1985). Phytochrome is a light receptor that converts an environmental signal into a developmental response, and as such has been implicated in the control of a variety of developmental processes. In dark-grown plants, phytochrome is present in the form P_r, which absorbs red light. The P_r form is converted by red light into the P_{fr} form which absorbs far-red light and is biologically active. Phytochrome in this form causes, directly or indirectly, the activation of one or more transcriptional regulatory proteins through a signal transduction which is as yet unknown. In the case of PCR, phytochrome-activated transcriptional regulators are believed to repress the transcription of the gene (Hopkins, 1995).

Previously in Jean Haley's lab at Colby college, a maize nuclear PCR gene was isolated and sequenced in order to study its regulation during light-mediated chloroplast development. Heterologous primers derived from conserved regions of the barley and oat PCR cDNA sequences were used in the polymerase chain reaction to amplify a PCR-specific fragment from maize nuclear DNA. The maize PCR DNA was cloned and

used to screen a lambda phage genomic library. A number of putative PCR clones were retrieved and one was sequenced. The nucleic acid sequence of the maize coding region was about 90% similar to wheat, barley, and oat cDNA sequences, and about 70% similar to pine cDNA and pea genomic sequences.

The promoter region of the sequenced genomic clone was isolated and inserted in front of a β-glucuronidase (GUS) reporter gene in a plasmid vector to be used in a microprojectile bombardment transient expression assay. A reporter gene allows the activity of a promoter to be tested under various experimental conditions by "reporting" its activity through the production of a product that is either colored or fluorescent. GUS is a widely-used reporter gene in plants because the GUS gene is normally absent in plant tissues, in contrast to its presence in vertebrates and many microorganisms, and there are typically no detectable background levels of β-glucuronidase activity in most higher plant cells (Jefferson, 1989). Also, expression of the GUS gene can be measured by fluorometric, spectrophometric, or histochemical assays depending on the substrate used. In this study the colorigenic substrate 5-Bromo-4-chloro-3-Indolyl-β-D-Glucuronic acid (X-glucuronic acid) was used in a histochemical assay to produce a blue product that accumulated in cells expressing the GUS gene. Various regions of the PCR promoter were cloned, attached to a GUS reporter gene, and inserted into a plasmid vector. The plasmid constructs were precipitated onto tungsten particles and bombarded into intact dark-grown leaf cells, where expression of the endogenous PCR gene was known to be high.

The blue "spots" (representing cells filled with the blue GUS enzyme product) were assayed in seven to ten day old dark-grown maize seedling

leaves that had been bombarded with chimeric plasmid DNA containing either the forward or the backward orientation of a 1.7 kb PST I- PST I PCR promoter fragment placed in front of the GUS reporter gene. We were looking for the presence and activity of controlling elements of the gene that would be found upstream of the TATA box at the start site of transcription, and that would maximize reporter gene expression in the leaf cells. Nested deletions of the PCR 1.7 kb fragment cloned in pUC18 were prepared using an "Exo-Size" kit (New England Biolabs) to ensure that the integrity of the promoter sequence was not lost in the cloning procedure. The maize genomic library was then further screened with an 800 bp maize nuclear coding region to find another clone containing a possible maize promoter region which would be used in similar transient GUS expression assays.

MATERIALS AND METHODS

Growth of maize seedlings:

Seeds of maize (*Zea mays*) were soaked overnight in distilled water. The seedlings were grown in the dark on moist vermiculite at 26 °C for 7-10 days. At this time their second leaves were harvested under a dim-green safelight to be used in the transient expression assays.

Plasmid DNA purification techniques:

Plasmid DNA containing an ampicillin resistance marker was transformed into competent TB1 *E. coli* host cells and plated onto LB Amp (100mg/ml) plates. The plates were inverted and incubated ovemight at 37°C. Single colonies were then inoculated into 3ml of LB medium with 100 µg/ml ampicillin and grown up overnight in a 37 °C shaking incubator. The 3 ml

overnight cultures were spun at 13,000 rpm for 30 seconds, the supernatant was discarded, and the pellets were resuspended in a cell resuspension buffer. The bacterial cells were lysed with an alkaline lysis solution to release the plasmid DNA which was then purified using a Prep-A-Gene kit (Bio-Rad). When greater amounts of DNA were needed, half of the 3 ml overnight culture was used to inoculate a 100 ml liquid culture, and plasmid DNA was subsequently prepared by alkaline lysis and CsCl density centrifugation (Sambrook et al., 1989).

Preparation of tungsten particles:

Tungsten particles (1.1 μm diameter, Bio-Rad) were prepared in 50 mg lots in sterile microcentrifuge tubes. The particles were sterilized with 500 μl of 95% ethanol for 20 minutes, and then were washed four times with sterile water by repeated centifugation and resuspension by vortexing. They were resuspended in a final volume of 500 μl sterile RO water. The particles in this form could be stored frozen at -20° C for up to a week.

Determination of optimum parameters for microprojectile bombardment:

Because plant cells have tough cell walls containing cellulose, it is difficult to get DNA inside intact cells (Fig. 1). The microinjection technique that is used to transform animal cells would not work. For that reason a simple and inexpensive particle acceleration device has been designed for direct delivery of DNA into plant cells.

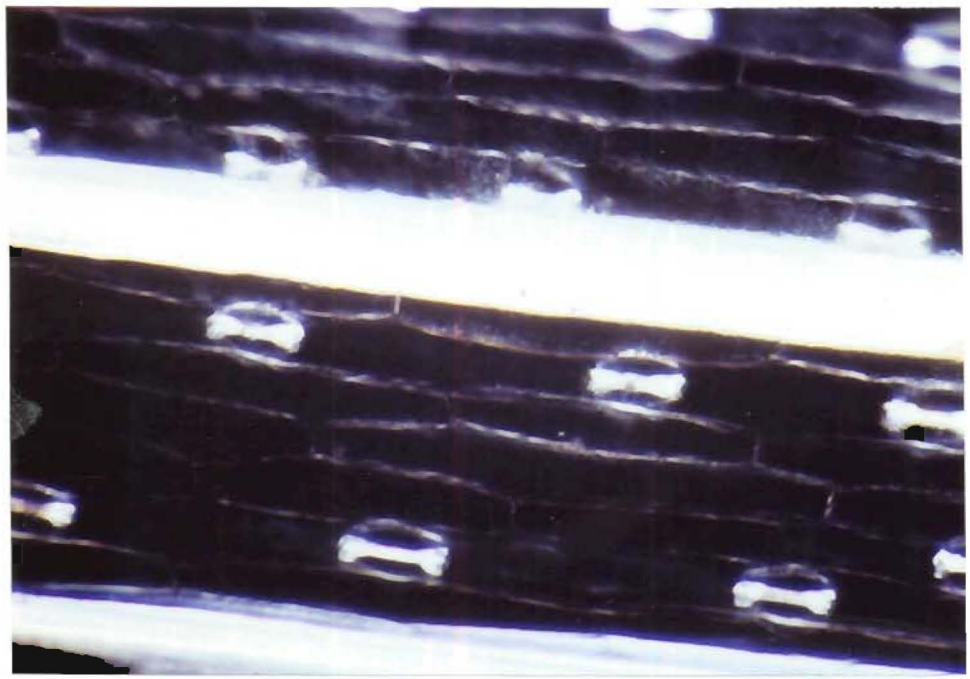


Figure 1. Maize seedling leaf under polarizing light. Guard cells and stomata are clearly visible. Along the veins there are supportive fiber cells surrounded by thick cellulose cell walls.

The Particle Inflow Gun (PIG) is based on the acceleration of DNA-coated tungsten particles directly into target cells in a low pressure helium stream and has resulted in high levels of transient expression of the β-glucuronidase gene following bombardment (Vain et al., 1993). The gun is connected to a helium tank, and a burst of helium is used to propel the tiny (1.1 μm diameter) tungsten particles into the leaf cells (Figs. 2 and 3).

A timer relay-driven solenoid provides consistent particle accelerations by controlling the amount of helium released at each burst. Further control is provided by using a prechamber upstream of the solenoid. By reducing and controlling the amount of helium burst used to accelerate the particles, the risk of tissue damage or displacement can also be

reduced. A vacuum chamber was also needed to decrease the drag on the particles to again reduce tissue damage. Before the actual experiments could be undertaken, the most effective parameters for the "shoot" had to be determined.

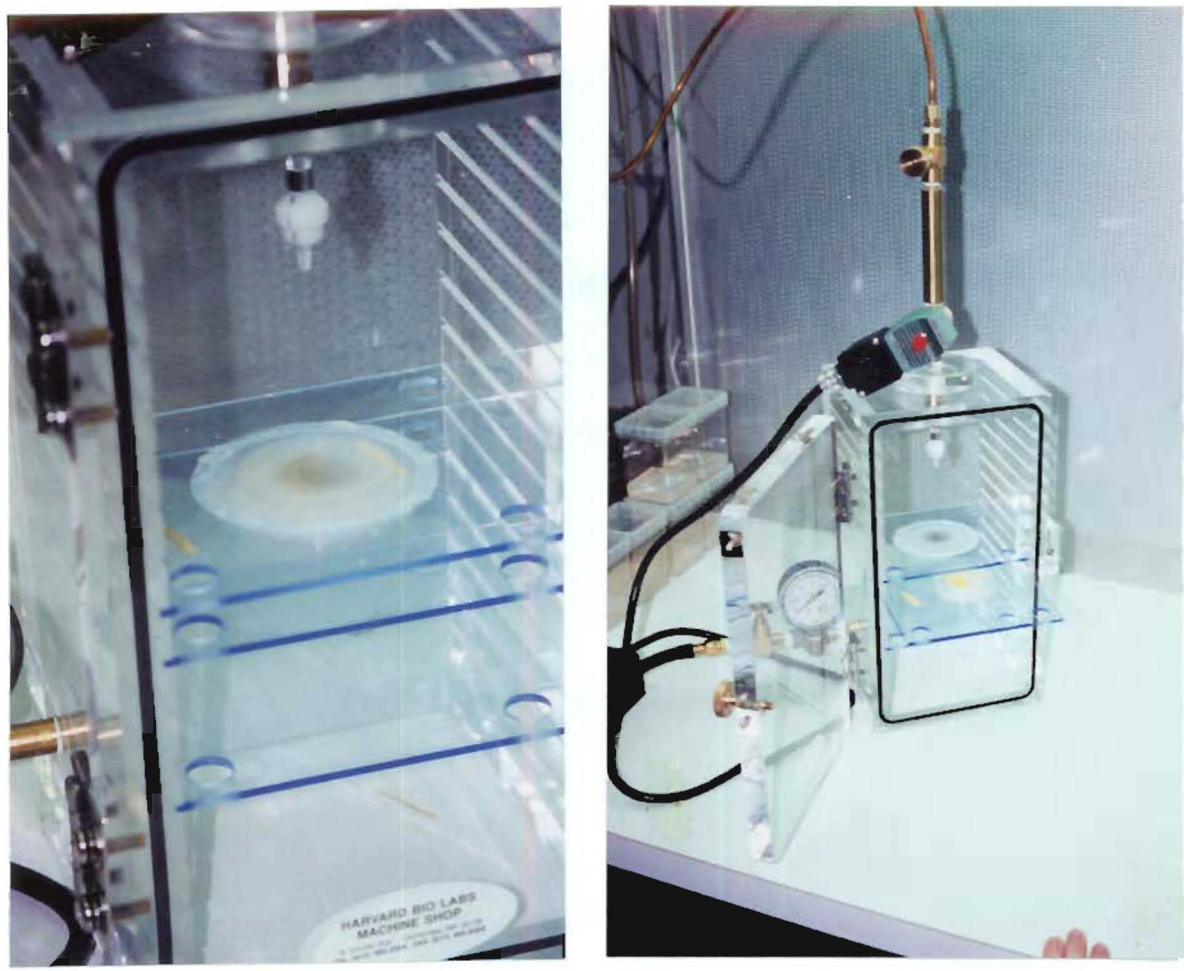


Figure 2. The Particle Inflow Gun (PIG) made by the Harvard Biolabs Machine Shop. It is attached to a helium tank and has a vacuum chamber in which the samples are bombarded with DNA-coated tungsten particles.

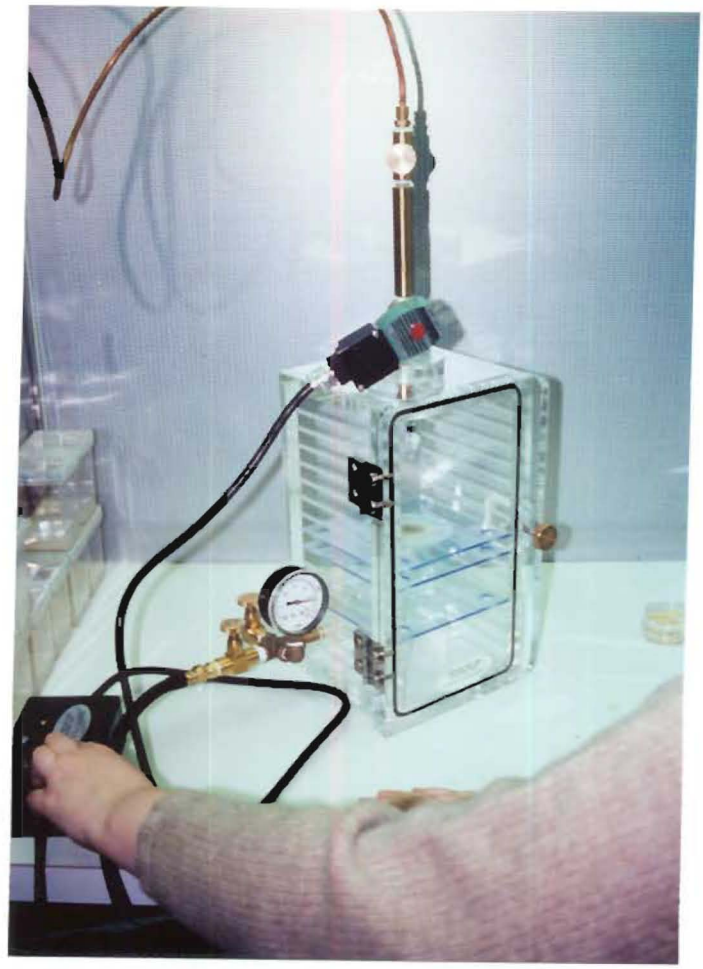


Figure 3. The solenoid is a small box attached to the PIG. When the timer-relay button is pushed, the burst of helium contained in the pre-chamber accelerates the tungsten particles, bombarding them into the maize leaf tissue.

Using the plasmid pBI221 (Clon-tech), which contains the 35S promoter from the cauliflower mosaic virus that is known to drive expression of the GUS reporter gene (Fig. 4), a series of bombardments were performed to determine if GUS expression in maize seedling leaves could be achieved.

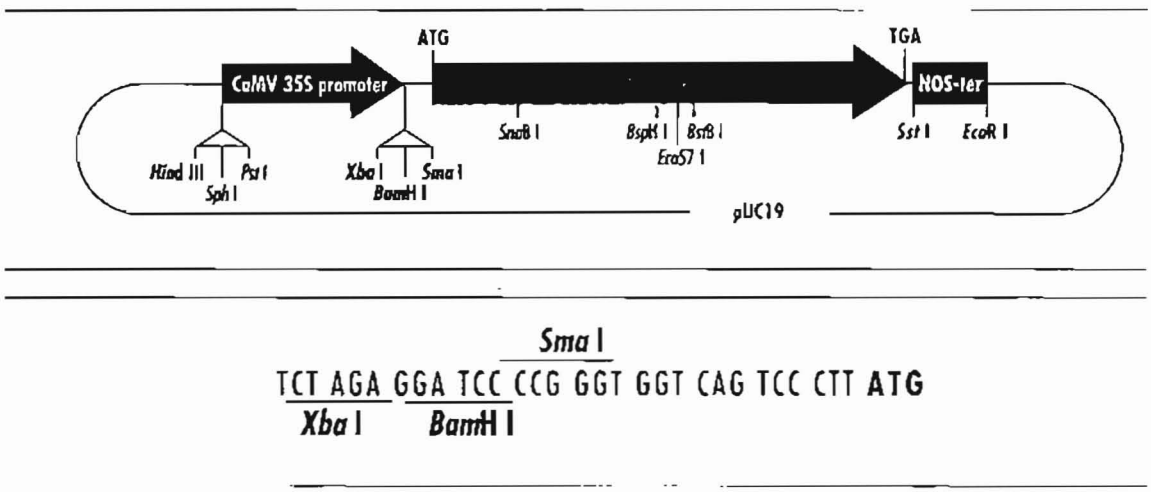


Figure 4. Plasmid pBI221 contains a 35S CaMV promoter, a β-glucuronidase cassette, and a NOS terminator region in a pUC 19 vector.

The leaf tissue from 7 to 10 day-old dark-grown corn seedlings (Fig. 5A) was harvested under a green safelight and placed on Murashige & Skoog (MS) medium containing 2% sucrose, 0.2 M sorbitol, and 1.5% agarose (pH 5.6) for support during the shoot, and to keep the tissue healthy for the subsequent incubations. The rolled leaves were flattened and spread onto the agarose surface with a bent-tip forceps rolled-side down so that the lower epidermis was facing upward, creating a continuous layer of leaf tissue over the entire 60 mm X 15mm petri plate (Fig. 6).

Figure 5. Greening of dark-grown maize seedlings when exposed to light. (A) Seven day old dark-grown seedlings that were used in transient microprojectile bombardment expression assays. Seedlings that have never been exposed to light have a yellow appearance. (B) After being exposed to 4 hours of light, some green pigment is visible in the seedlings. (C) After 16 hours of exposure to light, the seedlings are almost entirely green.

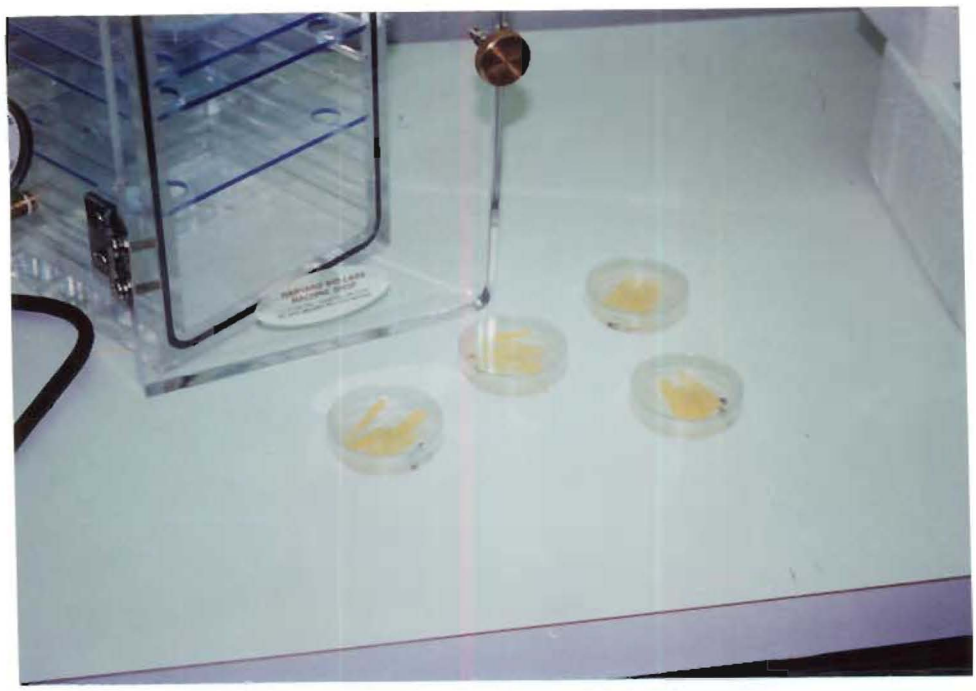


Figure 6. Maize seedling leaves were spread rolled-side down onto an MS agar medium prior to bombardment to provide support for the "shoot".

Five µg of plasmid DNA (purified from a CsCl density gradient centrifugation and dialyzed overnight in a sterile 10mM Tris, 1 mM EDTA buffer using a Slide-A-Lyser cassette [Pierce]), was precipitated onto tungsten particles in the presence of 1M $CaCl_2$ and 10 mM spermidine. After the particles were chilled on ice for 5 minutes, 50 µl of the supernatant was collected and discarded. The remaining mixture was kept on ice and provided enough volume for 5 shots, although only four shots were routinely used (Vain et al., 1993). The particles were finger- vortexed and 2 µl was quickly added to the filter syringe to avoid clumping (Fig. 7).

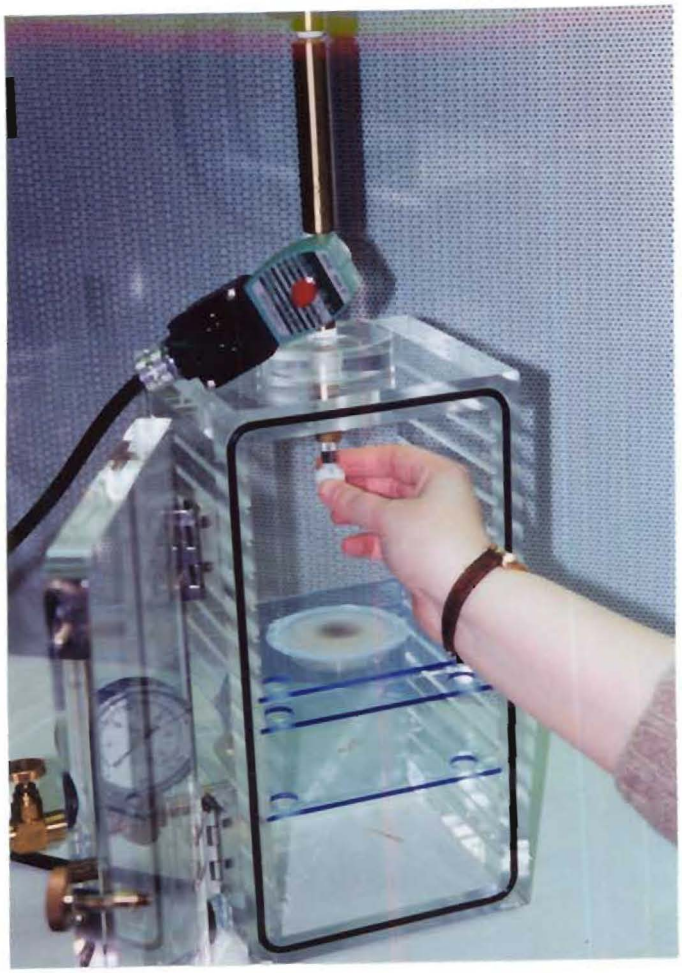


Figure 7. DNA-coated tungsten particles were added to the syringe filter on the PIG. When helium is released from the pre-chamber, the particles are accelerated into the maize leaf tissue so that the DNA can enter the intact cells.

Several shots were performed at various helium pressures to determine the appropriate amount of helium needed for maximum expression. The distance from syringe filter to baffle was also varied. A 500 µm nylon mesh baffle was placed over the tissue to disperse the tungsten particles over a larger leaf area while at the same time reducing tissue

displacement. Distance from baffle to tissue was kept constant at 1.5 cm. During the first series of shots, the tissue was partially blown off the agar by the force of the helium burst, but this problem was soon alleviated by decreasing the hardness of the medium slightly, from 1.5% agarose to 1.2 % plant tissue culture agar. Some leaf tissue was vacuum- infiltrated in 0.2 M sorbitol, or 5%, 10%, and 20% sucrose prior to bombardment to see if expression could be maximized that way.

After each bombardment, the tissue was left on the MS agar surface and was stored at 28 °C for 24-48 hours to give the cells adequate time to express β-glucuronidase. The colorigenic substrate 5-bromo-4-chloro-3-indolyl β-D-Glucuronic acid (X-Gluc) was used for the histochemical localization of GUS activity. This colorless substrate produces a blue indigo dye precipitate at the site of enzymatic cleavage. Color formation requires three separate reactions: after enzymatic turnover, the released indoxyl derivative dimerizes and is then oxidized to the final indigo dye (Gallagher, 1992). The X-Gluc substrate was dissolved at a concentration of 2-10 mM in a 0.1 M sodium phosphate buffer (pH 7.0) containing 10 mM EDTA and 0.5 mM each of potassium ferrocyanide and potassium ferricyanide. Following incubation at 28 °C, bombarded tissue was vacuum-infiltrated for several minutes with X-Gluc developing solution to help the liquid penetrate inside the tissue, and then further incubated in the developing solution for 24 hours at 37° C. The developing solution was removed at the end of this incubation, and the tissue was rinsed with 0.2 sodium phosphate buffer (pH 7.0) before being stored at room temperature in 70% ethanol to both de-colorize and preserve the leaves. This tissue was inspected with a dissecting microscope to detect cells that expressed GUS, visible as blue "spots". It was also noted if the blue spots were elongated rectangles or if

they were round in shape, as a way of determining what types of leaf cells were expressing the GUS reporter gene. The elongated cells are epidermal cells and the round cells are mesophyll and perhaps bundle-sheath cells that are located in the middle of the leaf underneath the epidermal layer.

Another plasmid was also used in developing a positive control for experimental bombardments. As illustrated in Figure 8, the plasmid BC17 (Ciba-Geigy) contains two regulatory genes that trigger anthocyanin production. Anthocyanin is the red pigment seen in autumnal maple leaves, but not normally expressed in maize leaves.

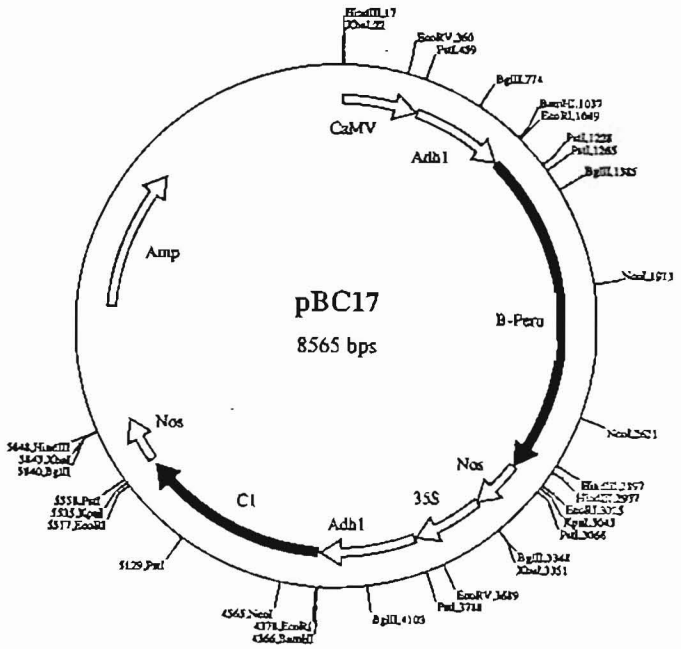


Figure 8. Plasmid BC17 contains two regulatory genes for anthocyanin production. When both genes are expressed, the red anthocyanin pigment is produced and it accumulates in the cell vacuole.

In maize, co-expression of both genes is necessary for the pigment to be produced. This was a good control to use because only intact cells will express the colored product and no substrate is needed for expression to be

visualized. Plates could be stored at 28 ° C for 48 hours and expression of the gene products seen in individual cells under a dissecting microscope. The shoot took place in the light because this is not a light-sensitive gene expression.

Construction of chimeric clones:

The 4.5 kb plasmid pTM44a (Figs. 9 and 10) was digested with the Pst I restriction enzyme at 37° C for two hours.

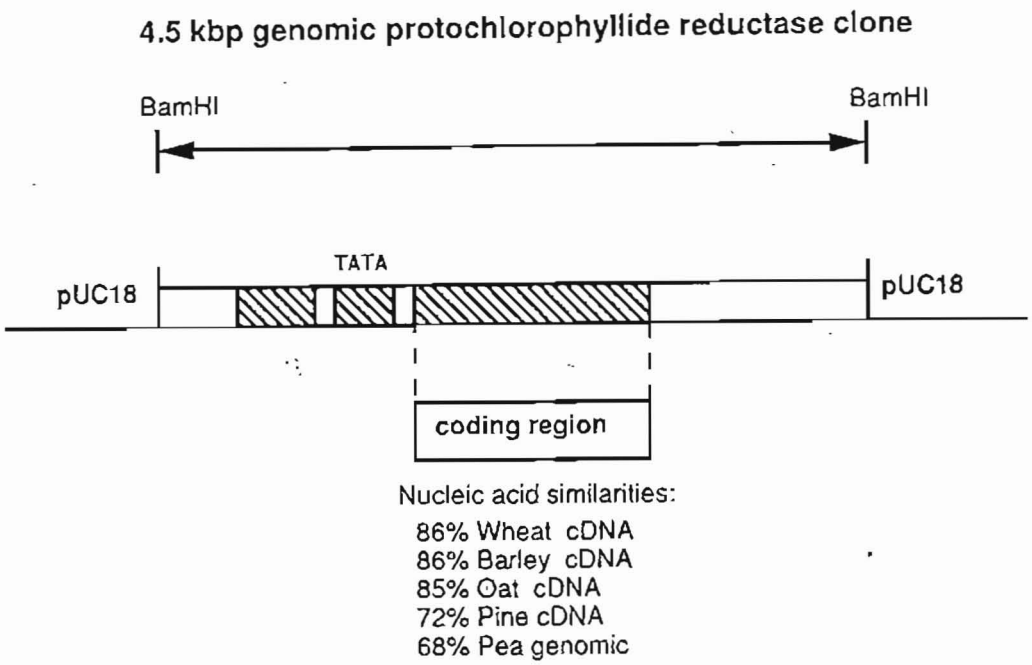


Figure 9. Restriction map of the 4.5kb genomic PCR clone pTM44a.

```
LOCUS       PTM44A -FO    282 BP DS-
BASE COUNT       60 A      97 C      72 G      50 T       3 OTHER
ORIGIN      POSITION 1 OF pTM44A -forward
        1 ATGTGAAGGT CCCGGCCTAG GTGACCGTGC CAANAATATC CCGCGGGAAA ATATCGTNAA
       61 GAAATCCGGG CCAGCCGCTG CGATTGGGTG CGGAGGGTCC ACGCTGCCAA AGGGCCCACC
      121 CCCTTATCCT ATCTTCGTGG CGCTCCTCGC TCCTCGCCCC GGGTATATAA GTACCGCNGC
      181 GAGCTCGTCG CCTCCGTCGA ACACAGAGTC ACTTCGCCAC GAACAAAAGC GCATCGATCT
      241 CGCTGTCGTC ACTCCTCGTC ACCCAGCCAC GAACAGAGGC AC
```

Figure 10. Partial sequence of the 1.7 kb fragment cloned from pTM44A. Note that there is a TATA box and a potential CAAT box.

Pst I cut the plasmid in two places, once in an internal site and once in the multiple cloning site (MCS), dropping out a 1.7 kb fragment. The digestion was electrophoresed on a 1% low-melt TAE agarose gel next to a 1 kb ladder and the fragment was isolated and purified from the gel using a PCR "magic" prep kit (Promega). The insert was cloned into the Pst I site of a GUS reporter gene vector that was engineered by Alice Cheung (Dept. of Biology, Yale University) by removing the β-glucuronidase cassette from pB101.1 (Clon-Tech) with a HindIII-EcoRI double digestion and inserting it into the MCS of PUC19 (Fig. 11).

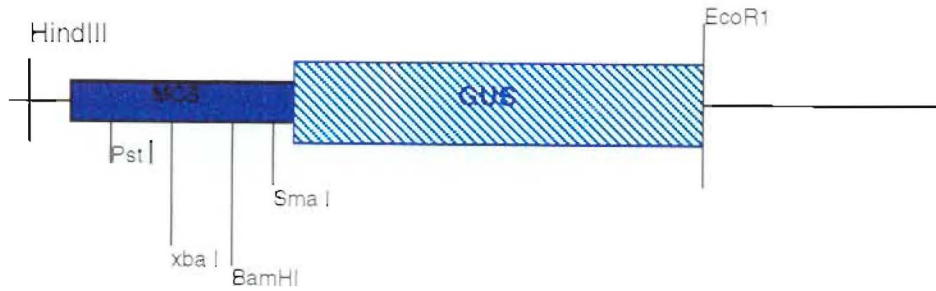


Figure 11. The GUS Expression Vector (GEV) consists of a β-Glucuronidase cassette in a pUC19 vector. The GUS cassette is located behind the Multiple Cloning Site where the PCR promoter constructs were inserted.

The GUS expression vector (GEV) was linearized with Sma I at room temperature and treated with calf intestinal alkaline phosphatase to prevent the recircularization of the cloning vector by removing the 5' phosphates which are required by ligases. Dephosphorylated vector cannot self-ligate, decreasing the background in cloning experiments. Samples of the insert and vector were electrophoresed on a 0.7% TAE agarose gel to determine relative concentrations of each, and then insert and vector were added to a 20 µl ligation mixture containing DNA ligase in a 3:1 ratio of insert:vector. The reaction mixture was incubated at 12° C overnight along with a control containing vector only, and the following day both were transformed into competent TB1 *E.coli* host cells. The plasmids contained an ampicillin resistance marker, so the cells were plated out on LB plates containing ampicillin and incubated at 37 °C overnight. Colonies grew up on both sets of plates indicating that the vector had self-ligated. Only 20 colonies from

the ligation plates with both insert and vector were screened using a rapid boil mini-prep (Sambrook et al., 1989). The plasmids were digested with Pst I to determine if they contained one or more inserts. The plasmids that contained only one insert were then digested with Sma I to check the orientation of the insert.

The same procedure was repeated with a PstI-BamHI fragment of pTM44a. The only difference in protocol was that Klenow enzyme had to be used to fill in the 3' end near the BamHI site so that vector and insert could be ligated with blunt ends (Sambrook et al., 1989).

Tungsten particle bombardments using the chimeric DNA:

For transient expression assays, 5, 10, 30, and 50 µg of each type of cloned and CsCl-purified DNA was precipitated onto tungsten particles and bombarded into 7 day old dark-grown maize seedling leaves. Separate syringe filters and baffles were used for each type of DNA to avoid cross-contamination. Both the tissue bombardment and the 28 °C incubation were performed in the dark with a green safelight. The incubation at 28 °C was performed at 24, 48, and 72 hours to give the gene product variable lengths of time to be expressed in the bombarded leaf cells. The amount of GUS expression was monitored in bombarded leaves after they were exposed to the X-Gluc substrate in developing solution for 24, 48, 72, and 96 hours. The supernatants from tungsten particle preparations containing 10, 30, and 50 µg of DNA were saved and electrophoresed on a 1% TAE agarose gel to monitor DNA absorption by the particles.

"Exo-Size" deletions sequencing:

The 1.7 Kb PCR promoter fragment was inserted into a pUC 18 vector that had been linearized with a Pst I cleavage. The clones were screened for both orientations and DNA of each type was bulked up in 100 ml liquid culture and then purified by alkaline lysis and CsCl density centrifugation. Following the protocol given in an "Exo-Size deletions kit (New England Biolabs) instruction manual (Fig. 12), target DNA was digested with restriction enzymes to yield an unprotected 5' end for exonuclease digestion and a protected 3' end to prevent the cloning vector itself from being digested.

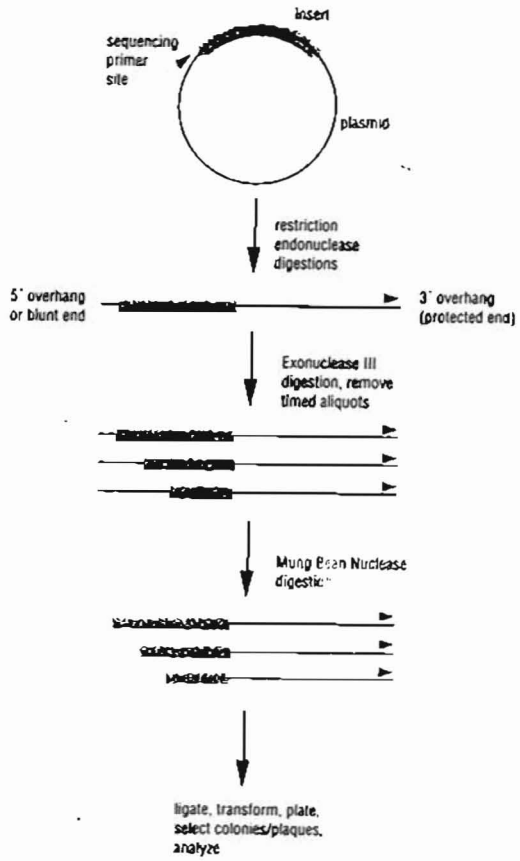


Figure 12. Protocol for creating exonuclease III deletion products, according to the "Exo-Size" (New England Biolabs).

Exonuclease III digestion started at the 5' end. Samples were removed every 10 seconds with 5 aliquots being added to each microcentrifuge tube. The deletion products were then treated with mung bean nuclease to remove single-stranded tails left by the exonuclease III digestion and were subsequently self-ligated. The ligation mixture was transformed into competent TB 1 host cells which were screened for appropriately-sized deletion products. Deletions differing in series by 200 bp could then be sequenced.

RNA blot analysis:

Total RNA isolated from dark-grown 7 day old maize seedling leaves that were exposed to light for 0, 3, 9, and 20 hours was electrophoretically separated on a 1.2% MOPS-formaldehyde denaturing gel. The gel was blotted to Zeta-probe (Bio-Rad) nylon membrane and hybridized overnight with a 1.3 kb 3' end-specific maize PCR probe that was purified from a 1% low melt agarose gel and radiolabeled with [^{32}P] dCTP. The membrane was then washed two times with 2X SSC/ 1% SDS and two times with 0.1X SSC/1% SDS. Each wash lasted about 10 minutes and was performed to remove background counts so that only the signal remained. The membrane was then sealed in a fresh bag and exposed to X-ray film overnight.

Maize genomic DNA clone analysis for another PCR gene:

A maize genomic library in Charon 4 λ bacteriophage was screened with a maize PCR coding region probe, putative clones were retrieved and isolated, and stock cultures of each made up (Jean Haley, personal communication). Stock culture of one λ phage clone, arbitrarily designated

clone 20, was added to LE392 plating bacteria and then plated out in top agarose on LB plates (Sambrook et al., 1989). The plates were incubated at 37 °C overnight to generate phage plaques. The plaques were harvested into SM buffer with 1 drop chloroform added per every 1 ml of SM. The DNA was purified from the phage using cut-off pipet tips to avoid shearing the DNA (Wizard λ phage Prep Kit, Promega), and it was then digested with various restriction enzymes and electrophoresed on a 1% TAE agarose gel. The gel was blotted onto a nylon membrane and hybridized with a radio-labeled 800 bp maize PCR coding region probe. Fragments hybridizing to the probe would then be isolated from the gel and cloned sequencing analysis and transient expression assays to determine if they do in fact contain another PCR promoter region.

RESULTS

Development of experimental system:

A helium tank pressure of 100 psi maximized GUS expression in terms of the number of cells that took up the DNA and expressed it, yielding a blue product when exposed to the colorigenic substrate. The initial shots were done at 60 and 80 psi, which probably accounted for the lack of expression in the tissue. In four different series of shots at 60, 100, and 120 psi, no GUS expression was visible at 60 psi, an average of 130 cells expressed a blue product at 100 psi and at 120 psi the average of blue GUS-expressing cells per shot was approximately 90. The experimental shoots were all performed at 100 psi and with a distance of 7.6 cm between syringe filter tip and baffle. Vacuum infiltrating leaf tissue with sorbitol, or with 5% or 20% sucrose, prior to the shoot greatly decreased expression,

while infiltrating with 10% sucrose somewhat increased expression, although not significantly. There was more GUS expression in the subepidermal cell layers than in the epidermal cell layer, with approximately 115 round blue mesophyll cells and 15 elongated blue epidermal cells per shoot at 100 psi, as an average of four shots (Figs. 13, 14, and 15).

Figure 13. GUS expression using pBI221 is evident in both epidermal (elongate) and mesophyll (round) cells.

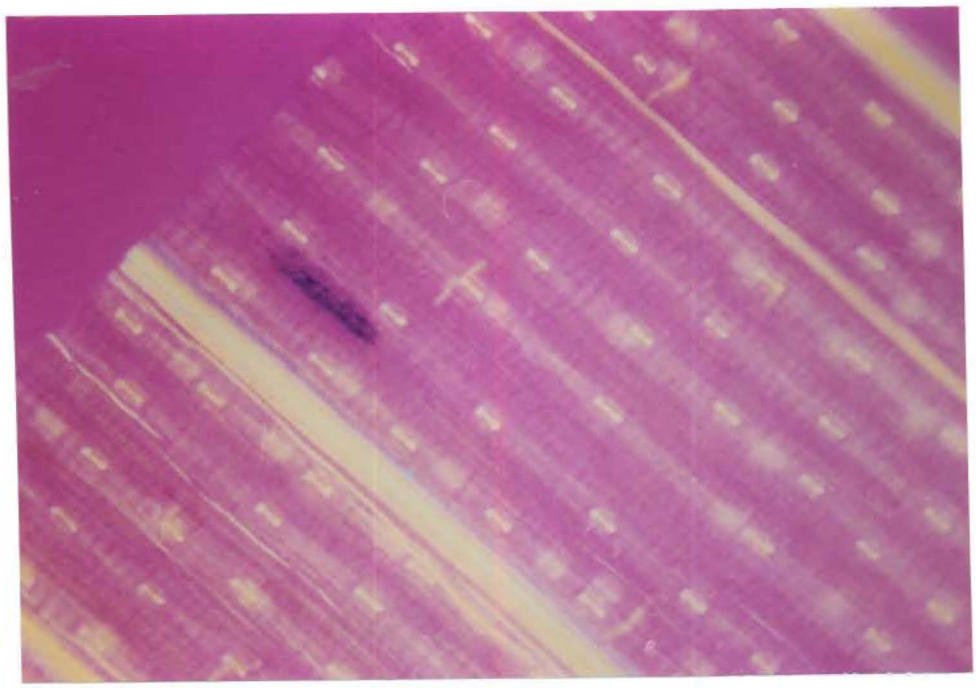


Figure 14. Close-up of blue "spot" under polarizing light with a red filter.

Figure 15. Close-up of pBI221 GUS expression in epidermal and mesophyll cells.

The expression pattern was the opposite with BC17 DNA. Most of the red pigment was found in epidermal cells (Figs. 16 and 17).

Figure 16. Anthocyanin pigment is produced when the two regulatory genes in BC17 are expressed.

Figure 17. Close-up of anthocyanin pigment filled epidermal cell.

Orientation of PCR promoter fragments in the GUS reporter vector:

Chimeric clones of the 1.7 kb PstI-PstI fragment of pTM44a were created in both the forward and backward orientations. When the fragment is cloned in the forward orientation, cleavage of the plasmid with Sma I produces a 6.1 kb piece and a 200 bp fragment (Fig. 18A). When the insert is cloned in the backward direction, the result of a Sma I digestion produces a 1.5 kb and a 4.8 kb fragment (Fig. 18B). Both orientations were achieved as shown in Figure 19.

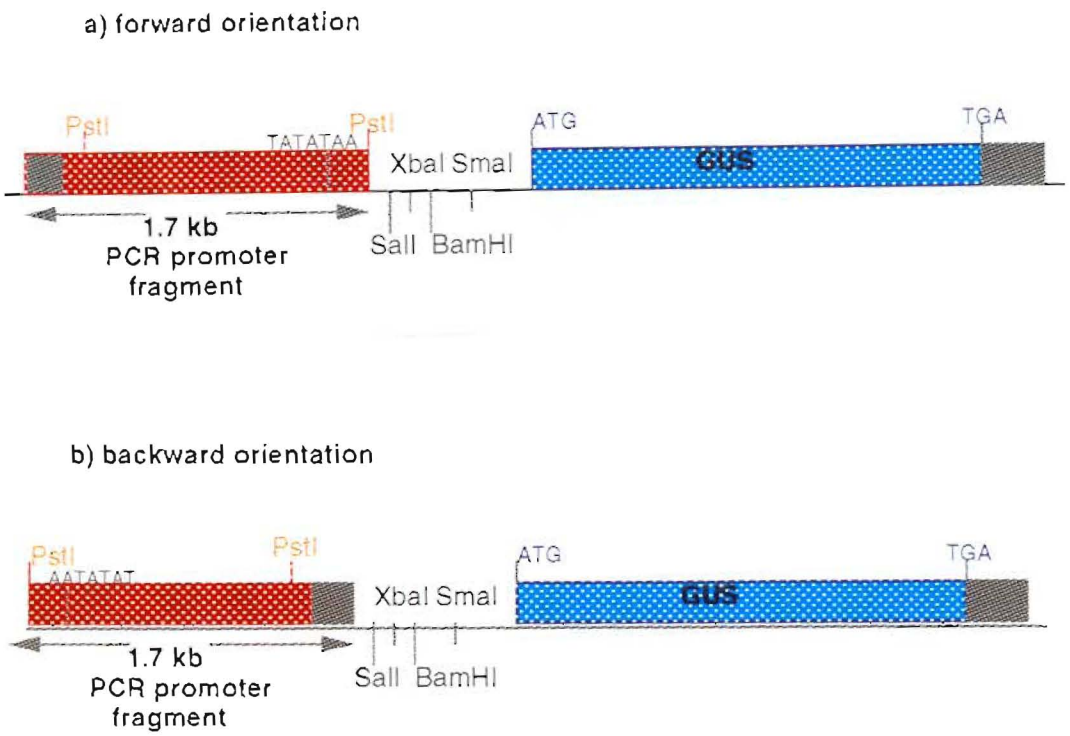


Figure 18. Restriction map of PCR-GUS constructs in pUC19 plasmid. A) forward orientation, designated 4a; B) backward orientation, designated 3b.

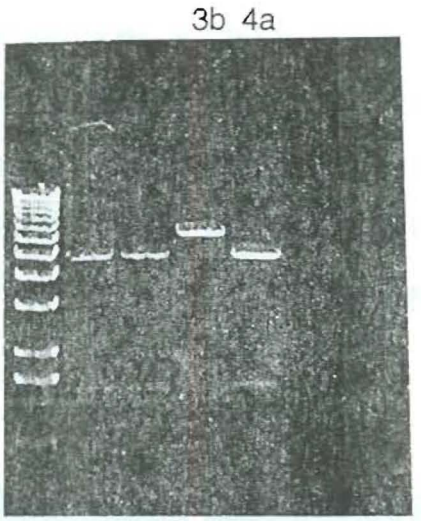


Figure 19. PCR-GUS constructs were linearized with Sma I and separated on a 1 % TAE agarose gel to determine the orientation of the insert.

One chimeric clone was screened that contained the 1.7 Kb Pst I-BamHI fragment (Fig. 20). However, no further analysis could be performed on that clone because we were unable to purify a high enough concentration of its plasmid DNA.

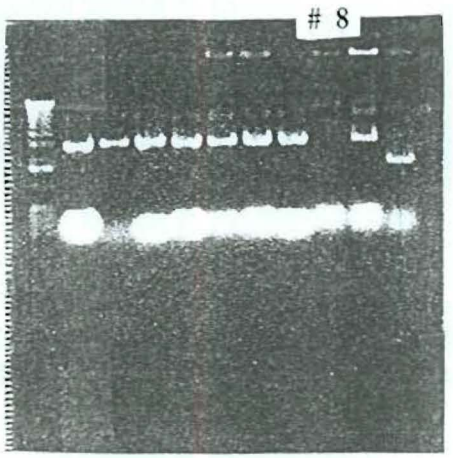


Figure 20. Pst I-BamHI 1.7 kb fragment was inserted into GEV. Rapid-boil mini-prep DNA was linearized with EcoR1 to determine which plasmids contained an insert. Plasmid #8 contained an insert

Transient expression levels of chimeric clones, 4a and 3b:

When tungsten particles coated with 5 µg of DNA were bombarded into the dark-grown maize seedling leaves and the tissue was developed for 24, 48, and 72 hours, no GUS expression was achieved using either clone. Slight expression (1-2 blue "spots" per shoot) was visible using 10 µg of the 4a cloned DNA when the tissue was left in the developing solution for more than 48 hrs (Fig. 21).

Figure 21. A blue "spot" was visible after a 7 day old dark-grown maize seedling leaf was bombarded with a tungsten particle coated with 4a DNA and subsequently exposed to X-Gluc for over 48 hours.

After 96 hours, it became very hard to tell what was a definite spot and what was bacterial contamination. There was no change in the level of transient GUS expression when the amount of DNA precipitated onto the particles was increased from 10 µg to 30 and 50 µg. When the supernatants from these three precipitations were separated on a 1% TAE agarose gel it appeared that the particles saturated after 10 µg, in other words there was DNA visible in the 30 and 50 µg supernatants. This could explain why there was little difference in expression between the three levels of DNA. No expression was visible in any of the bombardments using 3b, the backward orientation of the clone (Fig. 22).

Figure 22. No GUS expression was visible when particles coated with the chimeric clone 3b were bombarded into maize leaf tissue under the same conditions. The pale blue spot is probably an example of bacterial contamination.

When the 28 °C incubation was increased from 24 hours to 48 and 72 hours, there was no GUS expression visible when the developing time remained constant at 24 hrs. Again, slight expression was seen with the 4a clone when the developing time was extended to 48 and 72 hours.

"Exo-Size" deletions:

Deletions are visible in one of the two clones that was digested with exonuclease III prior to the DNA being self-ligated (Fig.23). No DNA was visible on the 1% TAE agarose gel loaded with the other clone. However, there were colonies on all the transformation plates, which can be screened in the future to determine if deletions are in 200 bp increments.

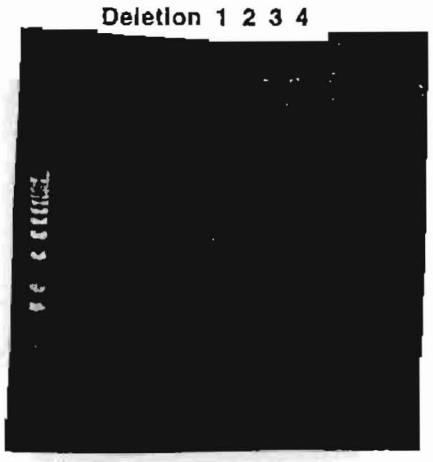


Figure 23. Sequential deletions of about 200 bp are visible in plasmid DNA digested with exonuclease III.

RNA blot analysis:

There was a lot of nonspecific background signal on the blot due to unknown technical problems, perhaps with probe purification or rinsing of the blot (Fig. 24).

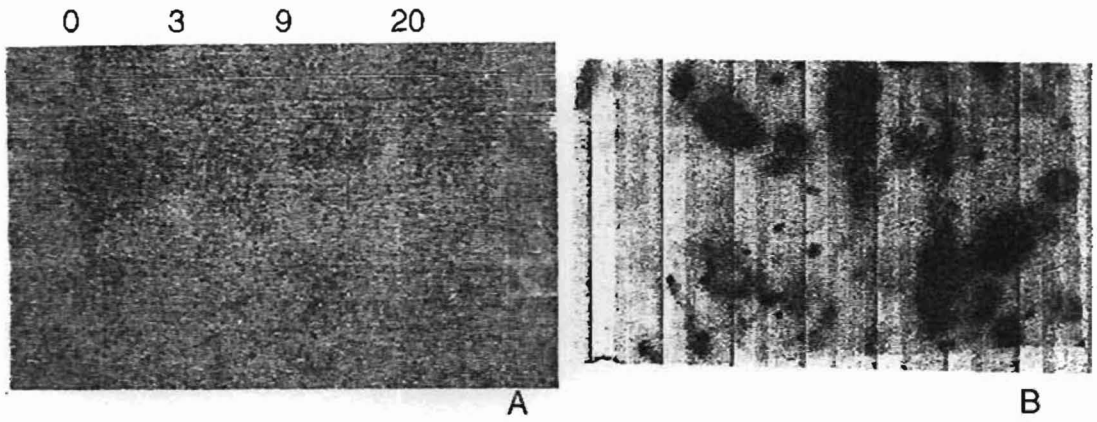


Figure 24. Northern blots illustrating the effect of light on PCR mRNA. (A) Earlier blot done by Earl Lewis using a coding region probe shows that light dramatically decreases the level of total PCR mRNA. (B) Blot with a 3' end specific probe has too much background to be analyzed.

DISCUSSION

Promoters are DNA sequences located upstream from a gene, on the 5' flanking side, which function in the regulation of transcription. Eukaryotic promoters are typically composed of several functionally different regions: a sequence that determines the transcription start site (TATA box) by serving as the binding site for RNA polymerase, and more distant regions that control the developmental, tissue-specific, or environmentally-responsive expression of the gene (Fig. 25).

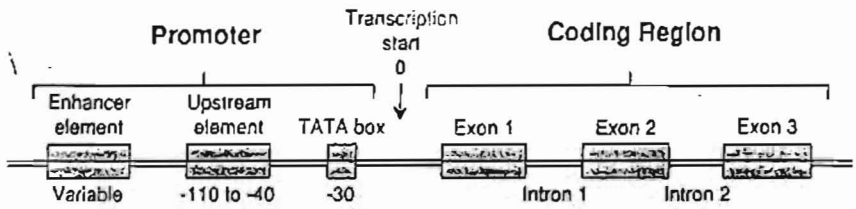


Figure 25. Schematic of a eukaryotic nuclear gene showing the promoter and coding region. (Murphy and Thompson 1988).

We subcloned the promoter region of a maize nuclear PCR gene, including 1.5 kb in front of (5' to) the TATA box and about 200 bp on the other (3') side of it, and ligated it to a GUS reporter gene to see if our promoter could drive GUS expression. We were looking for controlling regions located upstream of the site of transcription start that would

maximize GUS expression in dark-grown corn seedlings, but minimize GUS expression in greened leaves, a pattern that would mimic the *in vivo* developmental expression of the PCR gene. In this way, we could use the transient bombardment assay to locate light-responsive DNA control sequences. The slight expression of the clone in the forward orientation but lack of expression in the backward orientation suggests that this fragment of DNA may act as a classical promoter. The promoter would not be able to drive expression in reverse, because the TATA box would be upstream of the controlling regions.

Several factors could explain why only minimal expression, as determined by the lack of well-defined blue spots, was achieved. One of the problems with using a histochemical assay is that accurate interpretation of the data depends on the level of detection of the blue spots. We tried varying both the time that the promoter is able to express the gene product and the time that the gene product is exposed to the colorigenic substrate, X-Gluc, but still could achieve no more than minimal GUS expression. Moreover, the promoter could have limited activity, because it is not as strong a promoter as the 35S promoter from the cauliflower mosaic virus. It is also believed that PCR is encoded by a family of genes, so it is possible that we have cloned the promoter of one of the more weakly-expressed genes, or even a promoter of a PCR gene that is not very light-responsive. If there is a family of genes that encode PCR, each gene might be expected to be regulated differently. Previous work done by Earl Lewis indicated that total PCR mRNA decreases rapidly when dark-grown seedling leaves are exposed to light. In this study we attempted to use a 3' end-specific probe for our PCR gene to determine the light-responsive expression of this particular gene. Due to a high level of nonspecific background, we were

unable to get conclusive results concerning the gene's expression pattern. The RNA blot analysis needs to be repeated before more work can be done using this particular maize PCR gene. By rescreening the genomic library and repeating the transient microprojectile bombardment assays with 5' regions of other PCR genes, perhaps a stronger promoter can be located.

The only light the maize seedlings were exposed to during the bombardment experiments was a dim-green safelight, because under green light there is no significant conversion of phytochrome to P_{fr}. If phytochrome absorbs red light, it is converted to the biologically active form P_{fr} which causes the activation of regulatory proteins that inhibit PCR gene expression. In this work, we tried to determine the efficiency of a promoter region of a PCR gene, so we did not want to affect our results by using conditions that would cause a decrease in the already low expression of this promoter region. However, if a strong PCR promoter region is isolated it would be important to check its level of expression under red light which activates P_{fr} and reportedly inhibits the action of PCR by bombarding various stages of "greening" seedlings. Also if P_{fr} absorbs red light, it is converted to the inactive phytochrome derivative P_r, so it would be interesting to determine if expression after bombardment under far-red light would occur at a similar level as the expression achieved from bombardments that took place in the dark. This would indicate the extent of phytochrome involvement in the expression of PCR and chlorophyll synthesis.

CONCLUSION

The chimeric subclone of the PCR gene pTM44a, 4a, that contained the 1.7 kb promoter insert in the forward orientation did show weak expression of the GUS reporter gene in microprojectile bombardments performed entirely in the dark. No expression was visible using the subclone in the reverse orientation.

ACKNOWLEDGEMENTS

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