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Genetics

Maize sucrose synthase-1 promoter directs phloem cell-specific expression of *Gus* gene in transgenic tobacco plants

(tissue specificity/glucuronidase/plant promoter/anaerobic induction)

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ABSTRACT **Tobacco plants were transformed by using a chimeric gene construction, in which a corn sucrose synthase-1 gene (*Sh*) promoter was used to direct expression of the β-glucuronidase (*Gus*) reporter gene. Expression of *Sh-Gus* activity in these plants was found to be cell type specific. GUS activity was detected only in the phloem cells but not in any other cell types of vegetative tissues. In addition, *Sh-Gus* expression was found to be anaerobically inducible in tobacco roots. *Sh-Gus* was also expressed at high levels in the endosperm tissue of maturing tobacco seeds. We thus demonstrated that the corn *Sh* promoter can direct cell-type-specific and inducible expression in a heterologous dicotyledonous plant.**

Corn sucrose synthase 1 (*Sh*) gene, encoding sucrose synthase 1 isozyme (UDPglucose:D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13), is one of the few monocotyledonous plant genes whose biochemical function has been identified (1) and whose promoter sequence has been cloned (2–4). As sucrose synthase 1 isozyme is the primary enzyme of the various sucrose synthase isozymes, it is believed that it may play some important physiological role(s). The *Sh* gene was isolated from the corn *shrunken-1* locus, which is located on chromosome 9S (5). Enzyme activity studies with tissue extracts have shown that the sucrose synthase 1 isozyme in corn is expressed at high levels in endosperm, at a much reduced level in root, but not in green tissues or pollen (see ref. 6 for review). These results indicate that the *Sh* gene in corn is expressed in a tissue- or organ-specific fashion. Physiological studies have shown that *Sh* gene expression is anaerobically inducible in root and shoot tissues of etiolated corn seedlings (6, 7).

Using a chimeric gene construction, in which a corn *Sh* gene promoter is used to control the expression of the β-glucuronidase (*Gus*) gene, we transformed tobacco plants and examined in detail the expression of *Sh-Gus* activity in transgenic tissues. The observed cell-specific and inducible expression suggests the possible use of the *Sh* promoter for targeting chimeric gene expression to specific tissues in transgenic plants.

MATERIALS AND METHODS

Construction of Promoter Genes and Vectors. The maize sucrose synthase promoter and first intervening sequence was isolated from Pvu55, a maize *Pvu* I genomic fragment in pBR327 provided by B. Burr (2). The *Pvu* I site at −1347, relative to the start of transcription, was converted to an *Xho* I site by linker mutagenesis (W. Swain, personal communication). A 2430-base-pair (bp) *Xho* I/*Nco* I fragment, containing the *Sh* promoter, first intron, and translation start site was isolated and inserted into a promoter-less *Gus* expression vector (8). The resulting plasmid, designated pShGus, contains the *Sh* promoter from −1347 through the transcription start site, the first intervening sequence in the transcribed but nontranslated region, and the *Sh* gene translation start site at the *Nco* I site. pShGus was then cut with *Xho* I and inserted into an *Xho* I cut pTV4 vector (pTV4 contains a *nptII* gene for kanamycin selection and Ti border sequences; ref. 9) and was designated pTVShGus.

A soybean *rbcs* promoter was isolated from pSRS2.1 provided by R. Meagher (10). The *rbcs* promoter was altered to place an *Xho* I linker at about −1550 relative to the start of transcription and an *Nco* I site was placed at the translation start site at +45. The resulting 1595-bp *Xho* I/*Nco* I soybean *rbcs* promoter fragment was then inserted into a promoterless pCMC1100 vector at the *Xho* I/*Nco* I sites to form a soybean *rbcs-Gus-nos* 3′ gene fusion designated pCMC2100. pCMC2100 was then cut with *Xho* I and the entire plasmid was inserted into pTV4 as described above. A soybean heat shock (*hs*) promoter was cloned with the gene *hs6871* described by Schoffl *et al.* (11). This promoter was cloned by using polymerase chain reaction (PCR) primers to amplify the promoter fragment from soybean genomic DNA and resulted in an *Xho* I site at about −420 and an *Nco* I site (+100) at the translation start site. The 520-bp *Xho* I/*Nco* I promoter fragment was transferred to a *Gus* expression vector and then to a transfer vector as described above for pTVShGus.

Plant Transformation. We generated tobacco plants with the promoter expression DNAs described above both via *Agrobacterium* and electroporation. Tobacco stem sections were transformed with disarmed Ti plasmid via *Agrobacterium* infection as described (9).

Alternatively, tobacco mesophyll protoplasts were electroporated by standard cell culture manipulations (12). Specifically, we used the following conditions: 10 μg of plasmid DNA per ml, 200 μg of salmon sperm DNA per ml (sonicated), 2×10^6 cells per ml, 350 V/cm, 500 μF, 25 msec using a PDS (Prototype Design Services, New York) electroporation apparatus. Transformed cells or tissues were selected on medium containing kanamycin (100 μg/ml) and plants were regenerated (9). Transformed plants were grown under standard greenhouse conditions. Unless otherwise indicated, 2- to 3-foot-tall plants were used for assay of cell-type-specific expression of GUS activity.

Histochemical Staining of GUS Activity. Stem, leaf, root, and other parts of plant organs were cut from transformed plants. Thin (0.3–0.5 mm), cross tissue sections were hand cut and submerged in GUS reaction mixture as described (13). Tissue sections in GUS reaction buffer were vacuum infiltrated at 300 mmHg for 2 min. Tissues were incubated at 30°C for 1–8 hr, depending on the level of GUS activity expressed in test samples. Unless otherwise indicated, *Sh-Gus* tissues were stained for 8 hr, while *rbcs-Gus* and *hs-Gus*

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Abbreviation: PCR, polymerase chain reaction.

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tissues were stained for 1–2 hr. The reaction was stopped by fixing in phosphate-buffered formalin, and chlorophyll-containing green tissues were bleached with 75% (vol/vol) ethanol. Cellular location of GUS activity was observed by light microscopy. Photographs were taken with a Zeiss photomicroscope and Kodak Gold 100 film.

Anaerobic Induction and Heat Shock Treatment. Anaerobic induction of root *Sh-Gus* activity was performed as follows. R_1 progeny seeds of *Sh-Gus* transformed tobacco plants were grown for 4 weeks in the greenhouse (20 cm tall). Segments of main root (connecting to stem, ≈0.5 cm diameter) were cut off the plants and sliced longitudinally along the root axis to give two corresponding half-root samples. One set of half-root from each plant was submerged deep into 50 ml of MS medium in a 50-ml conical culture tube. Carbenicillin (100 μg/ml) and nystatin (100 units/ml) were added to the MS medium (14) to prevent growth of microorganisms. The submerged half-roots were vacuum infiltrated (300 mmHg; 2 min) to provide better anaerobic conditions for the test. The second set of half-roots was rinsed twice in MS medium and placed with the cut side down in a humidified Petri dish. After 2 days, tissue sections were cut and GUS activities in the corresponding two half-root samples from each plant were compared. For heat shock treatment, freshly cut stem and leaf segments (1 cm long) were submerged in MS medium (14) supplemented with 0.1 M glucose. Stem and leaf tissues were then transferred to the same medium preheated to 45°C and incubated at 45°C in a water bath for 5 min with occasional shaking. Test tissues were then transferred to a 25°C medium, held for 10 min, and heat shock treated again as described above. Heat shocked and control segments were then held in a humidified Petri dish for 16 hr before sectioning and assaying for GUS activity.

PCR Analysis. DNA from ≈10 mg of plant tissue was extracted, ethanol precipitated, and redissolved. One hundred nanograms of these crude DNA preparations and 20 pmol of each of the appropriate primers were used under standard PCR conditions in a Perkin–Elmer thermal cycler as described (15).

RESULTS

A simplified map showing the main features of corn *Sh* promoter, soybean *rbcs* promoter, and soybean *hs* promoter used in our *Gus* gene constructions is depicted in Fig. 1. The transformed tobacco plants and the R_1 progeny were grown in the greenhouse, various organs were harvested, and thin tissue sections were stained for GUS activity and examined by light microscopy.

Fig. 2*A* shows the expression of GUS activity observed in a young root section of *Sh-Gus* transformed tobacco plants. Clusters of cells that were stained blue were localized in phloem tissues. No activity was detected in xylem, pith, cortex, or epidermal tissues. At high magnification (Fig. 2*B*), the cellular localization of GUS activity was found to be highly specific. A group of four to six cells residing at the center of phloem tissue clusters are highly stained, and the immediate parenchyma cells show little or no stain. The remaining cell types of the vascular cylinder, including xylem fiber, xylem parenchyma cells, pith parenchyma cells, pericyle, and endodermis cells were not stained. At still higher magnification (data not shown), we observed that two to three pairs of sieve elements and companion cells were localized at the center of each phloem tissue bundle, and both cell types were similarly stained with high GUS activity. The surrounding parenchyma cells of the phloem tissues, as shown in Fig. 2 *B* and *D*, express little or no activity. At this level of cellular resolution, we thus have used the term "phloem cells" (including only sieve elements and companion cells) rather than phloem tissues to describe our results.

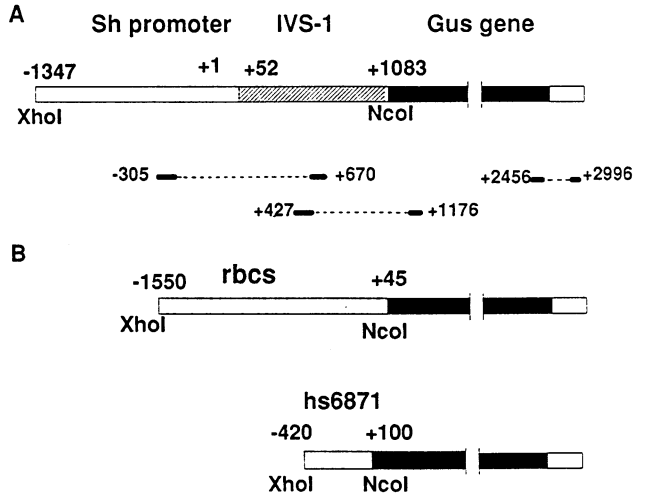

FIG. 1. (*A*) Maize *Sh-Gus* expression cassette. The location of the maize *Sh* promoter up to the translation start site at the *Nco* I site is indicated by the open box. The site of transcription initiation is indicated by +1. The first intervening sequence (IVS-1), which falls in the nontranslated leader sequence, is indicated by the hatched box. The *Gus* gene (solid box, truncated) and the *nos* 3′ poly(A) termination region are also shown. For PCR analysis, synthetic oligonucleotides were used as primers. The locations of the primers are shown by the solid bars (DR140, −305; DR139, +670; DR138, +427; DR72, +1176; DR57, +2456; KB71, +2996). The region of the *Sh-Gus* gene amplified by PCR is shown by a dotted line (see also Fig. 3). (*B*) Constructs of the upstream region for soybean *rbcs* and soybean *hs* promoters. Promoter fragments were ligated to the *Gus* coding sequence as transcriptional fusions.

Fig. 2*C* shows the expression of GUS activity observed in a stem section of *Sh-Gus* transformed tobacco plant. A special feature of tobacco stem is that it contains two groups of phloem tissues—namely, the internal and external phloems, which are located along the two sides of the xylem. Most other dicotyledonous plants only have external phloem. As shown in Fig. 2*C*, GUS activity is detected in both types of phloem cells but not in any other cell types of the stem, including epidermis, xylem, and ground tissue cells of pith and cortex. At higher magnification (Fig. 2*D*), the cellular localization of GUS activity was found again to be highly specific. The phloem cells were stained, whereas the adjacent phloem parenchyma cells and vascular cambium cells were not. Phloem cell-specific expression of *Sh-Gus* was also observed in leaf, flower, and fruit tissues. Fig. 2*E* shows that only phloem cells in midrib and lateral veins of leaf tissue expressed high levels of GUS activity, whereas no activity was detectable in other leaf cell types. Phloem cell-specific expression of *Sh-Gus* activity was also observed in flower and fruit tissues (Fig. 2 *F* and *G*, respectively).

In corn, the *Sh* gene is expressed at high levels in the endosperm of the immature kernal (1, 6). We have tested *Sh-Gus* expression in the endosperm of transgenic tobacco plants. Fig. 2 *G* and *H* shows that GUS activity could be readily detected in the thin endosperm tissue of immature tobacco seeds. In dicotyledonous plants, endosperm tissue in seed is formed after fertilization as a thin layer of nutritive tissue aligned in a concave fashion with the inner seed coat (16). The tissue is initially transparent and is later either absorbed by the developing embryo or pressed in between the cotyledon and seed coat in the mature seed. A tissue section was cut longitudinally through a maturing seed (Fig. 2*G*). It shows that a high level of *Sh-Gus* activity is expressed in tobacco endosperm tissue, but there is no activity in the cotyledon (Fig. 2*G*). Fig. 2*H* shows that, in a younger immature seed and at high magnification, GUS activity in young endosperm is readily detected at the cellular level. To

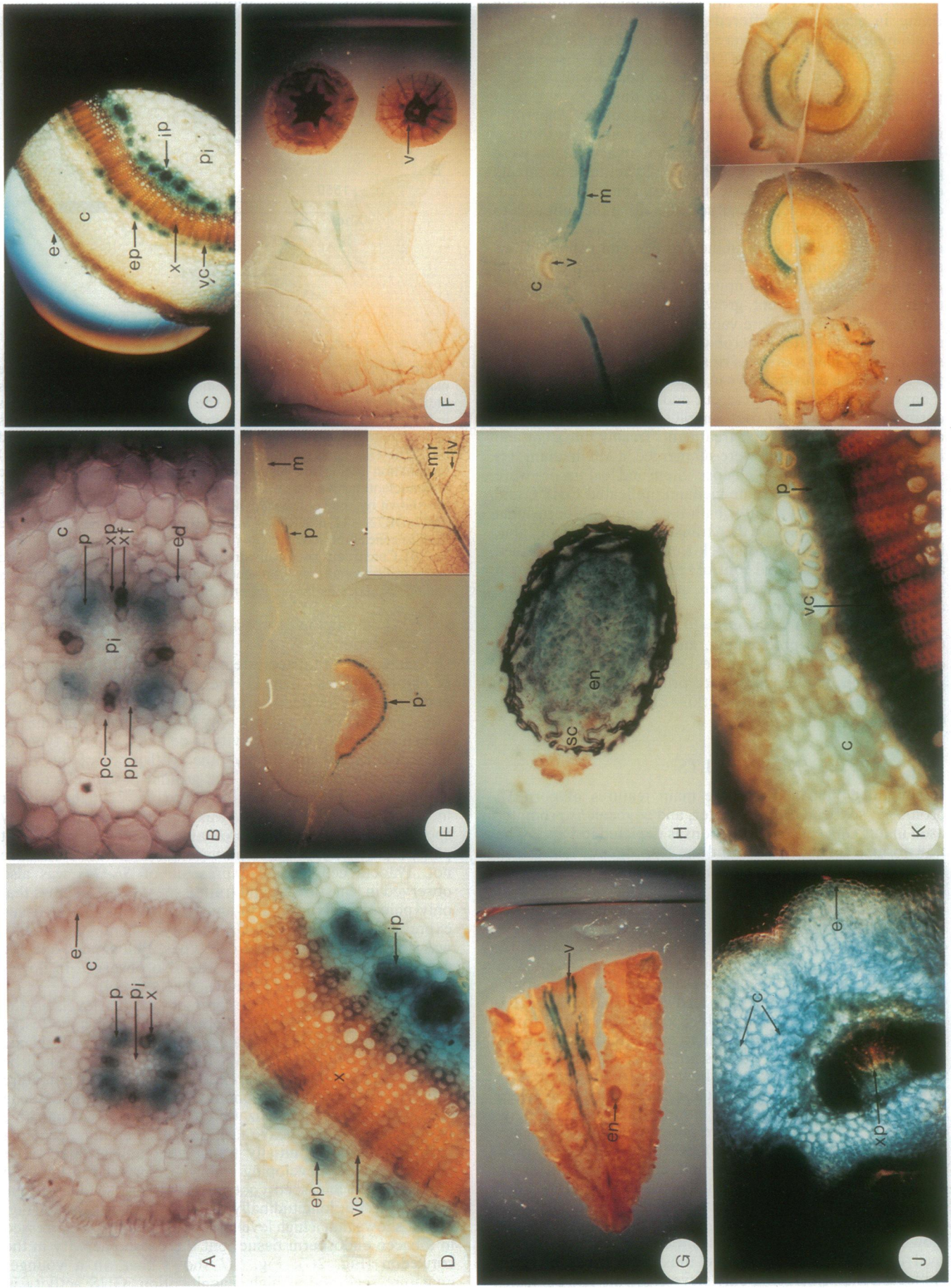

FIG. 2. *(Legend appears at the bottom of the opposite page.)*

better show the endosperm tissue, we cut the immature seed longitudinally into halves and gently teased out the embryo and cotyledon from endosperm and seed coat tissues. The focal plane of Fig. 2*H* was adjusted close to the bottom of the sliced half seed. In this case, the endosperm tissue was two to three cell layers thick and was lined with the seed coat. GUS activity was detected in small round endosperm cells but not in large polygonal seed coat cells. When *Sh-Gus* tobacco seeds were germinated in the dark for 4 days, enzyme activity could be detected only in the very fine veins extending through the root, hypocotyl, and cotyledon tissue, but not in any other tissues of the seedlings (data not shown). Hence, the pattern of phloem cell-specific expression of *Sh-Gus* activity, observed for mature transgenic tobacco plants, was conserved in young tobacco seedlings.

The same pattern of cell-specific expression for *Sh-Gus* in transgenic tobacco plants was observed for three independently transformed plants. Similar expression patterns were observed whether the plants were produced by electroporation or by *Agrobacterium* infection. In addition to regenerated R_0 plants, the selfed R_1 progeny (five plants for each transformant) were similarly tested for their GUS expression patterns and the same results were obtained as shown above. We therefore demonstrated that GUS expression dictated by the maize *Sh* promoter in transgenic tobacco plants was transmitted to the next generation.

Results showing localized GUS staining (Fig. 2 *A–G*) indicate that the maize *Sh* promoter directs phloem cell-specific expression of the *Gus* gene in transgenic tobacco plants. However, it is important to show that the results are not due to differential staining anomalies. For comparison, transgenic tobacco plants transformed with the same *Gus* gene but with different plant gene promoters (see Fig. 1) were tested under identical conditions for the GUS assay. Fig. 2*I* shows that tobacco plants transformed with soybean *rbcs* promoter express high levels of GUS activity in leaf mesophyll cells but not in phloem or other cell types of leaf tissues. Fig. 2*J* shows that, 16 hr after heat shock, tobacco plants transformed with the soybean heat shock promoter express high levels of GUS activity in virtually all cell types present in leaf tissues, including cortex, vascular cambium, xylem parenchyma, epidermis, and phloem cells. In stem, vascular cambium and phloem cells are apparently more responsive to the heat shock treatment than other cell types and were readily stained with GUS activity (Fig. 2*K*; see Fig. 2*B* for comparison). These results confirm that GUS enzyme produced in various cell types of tobacco tissues, including phloem, vascular cambium, xylem parenchyma, cortex, and mesophyll tissues, can be nondiscriminatively detected by our GUS activity assay. The experiments confirm that the phloem cell expression pattern observed for *Sh-Gus* transgenic plants is highly specific, is directed by the *Sh* promoter, and is not due to some unknown factors that may differentially affect activity staining in our GUS assay.

Expression of the *Sh* gene in young corn seedlings can be induced under anaerobic conditions (6). Springer *et al.* (7) have shown that the level of *Sh* gene transcripts increases 10 and 20 times in shoot and root, respectively. In *Sh-Gus* tobacco plants, we have observed that GUS activity is anaerobically inducible in root tissue. Using replicate half-root samples prepared from the root of the same plants, we showed (Fig. 2*L*) that root GUS activity is clearly induced under anaerobic conditions. With the short time period (≈30 min) used for GUS staining in this case, only a low level of activity was detected in the control (aerobic) root tissues, but high levels of enzyme activity were observed for the anaerobically treated half-root tissues. The expression of GUS enzyme remained phloem specific. When six R_1 progeny from the same transgenic parent were tested for anaerobic induction of *Sh-Gus* expression in root, four responded with strong induction (similar to Fig. 2*L*). The other two plants showed little observable differences in GUS activity in half-root samples that were treated with or without anaerobic induction conditions, suggesting that these plants were only weakly induced above the basal expression level found in roots. Overall, our results suggest that *Sh-Gus* activity in root phloem cells of transgenic tobacco plants is anaerobically inducible and the *Sh* promoter is apparently responsible.

PCR analysis was performed to provide molecular evidence that an intact *Sh-Gus* gene was introduced into tobacco plants. Fig. 1 indicates the positions of the primers used to detect the presence of each of the regions of the *Sh-Gus* gene. Primers DR140 and DR139 detected a 975-bp fragment spanning the transcription start site from −305 in the promoter to +670 in the nontranslated intron. Primers DR138 and DR72 detected a 749-bp region from within the intron to the 5′ coding region of the *Gus* gene. Primers DR57 and KB71 could detect a 540-bp region from the 3′ end of the *Gus* coding region into the *nos* 3′ terminator. These three sets of PCRs demonstrated that the promoter, intron, and *Gus* gene were intact. Fig. 3 shows the PCR results for two transgenic plants. Tobacco plant 3487 was produced via *Agrobacterium* infection, while tobacco plant 2713-14 is the R_1 progeny of plant 2713, which was produced via electroporation. The data demonstrated that transgenic plants recovered from different transformation methods contain intact genes and show the same expression patterns.

DISCUSSION

In this report, we demonstrated that the corn *Sh* gene promoter can direct highly specific, phloem cell-type expression of the *Gus* gene in transgenic tobacco plants. *Sh-Gus* was also found to express at high levels in endosperm tissue of immature tobacco seeds. In addition, the corn *Sh* promoter could be anaerobically induced in phloem cells of tobacco root tissue. Thus, the *Sh* promoter can target phloem cells as the tissue for foreign gene expression in genetically engineered plants. As phloem tissues of various plant organs are the target tissues for many plant pathogens, highly specific phloem expression of pathogen-resistant gene(s) via the *Sh* promoter may be utilized in disease control. It can also be used for anaerobic induction of foreign genes in root phloem cells.

Springer *et al.* (7) have demonstrated that expression of *Sh* gene at the transcriptional level is readily detectable in shoots of etiolated corn seedlings (including first and second leaves). A very low, but detectable, transcript level is found in mature green leaves. Upon anaerobic stress of young seedlings, the level of *Sh* transcripts increases 10–20 times in shoot and root, respectively. Our results in transgenic tobacco suggest that the expression of the *Sh* promoter is best described not

Fig. 2 (*on opposite page*). Histochemical localization of GUS activity in transgenic tobacco tissues. Expression of *Sh-Gus* gene in tobacco tissues: (*A*) Root at low magnification. (×4.) (*B*) Root at high magnification. (×15.) (*C*) Stem. (×4.), (*D*) Stem. (×15.) (*E*) Leaf midrib and mesophyll (*Inset*, part of a whole leaf). (*F*) Petal and ovary base. (*G*) Fruit and seed endosperm. (*H*) Immature seed endosperm. Expression of *rbcs-Gus* gene in tobacco tissue: (*I*) Leaf midrib and mesophyll. Expression of *hs-Gus* gene in tobacco tissues: (*J*) Leaf. (*K*) Stem. Anaerobic induction of *Sh-Gus* in root tissue of transgenic tobacco: (*L*) Half-root samples. (*Upper*) Anaerobic roots. (*Lower*) Control (aerobic roots). p, Phloem; ip, internal phloem; ep, external phloem; pp, phloem parenchyma; c, cortex; pi, pith; x, xylem; xp, xylem parenchyma; xf, xylem fiber; e, epidermis; v, vascular bundles; vc, vascular cambium; ed, endodermis; mr, midrib; lv, lateral vein; m, mesophyll; en, endosperm; sc, seed coat layers.

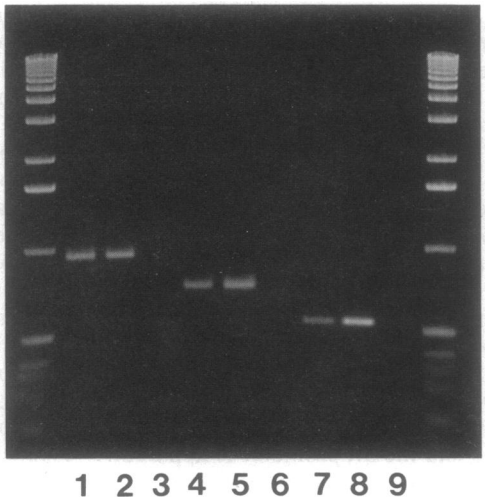

FIG. 3. PCR analysis to detect the *Sh-Gus* gene in transgenic plants. PCR was used to show that intact genes were transferred to the transgenic tobacco plants. Lanes 1–3, PCRs containing primers DR140 + DR139 and genomic DNA from Tob 3487, Tob 2713-14, and Havana 425 (nontransformed control), respectively. Lanes 4–6, reactions containing DR138 + DR72 and genomic DNA from Tob 3487, Tob 2713-14, and Havana 425, respectively. Lanes 7–9, reactions with DR57 + KB71 and genomic DNA from Tob 3487, Tob 2713-14, and Havana 425, respectively. Outer lanes are size markers.

at the organ level but at the cellular level. The *Sh* promoter is specifically expressed in phloem cells and it is the relative abundance of phloem cells in different organs that contributes to the organ-specific pattern one would observe by measuring *Sh* promoter activity in leaves, shoots, or roots. If one takes into account the relative abundance of phloem cells in these various organs, the pattern of expression we see in this system appears to be consistent with the expression observed in corn by Springer *et al.* (7). As shown in Fig. 2*E*, *Sh-Gus* expression is observed in leaf phloem cells. Since these cells account for <5% of the midrib cells and 1% of the leaf blade cells (estimated by microscopy), the leaf as an organ would be expected to show only low levels of *Sh* promoter expression. Therefore, when RNA, protein, or enzyme specific activities are measured from whole organ extracts, highly specific cell-type expression of a test gene could be drastically reduced or become nondetectable. Furthermore, efficiencies for tissue extraction are often found to vary considerably among different plant organs, this could further interfere with the biochemical assays for gene expression that is highly specific at the tissue or cell level.

Rowland *et al.* (17) showed very recently that the *Sh* gene transcripts are expressed in a cell-type-specific manner in corn root tissues. In their study, only the root tissues were tested. Expression was detected mainly in the vascular tissues, to a lesser extent in pith and epidermis, and not at all in cortex tissues. We have demonstrated that the *Sh-Gus* activity in transgenic tobacco is expressed only in the phloem cells of vascular tissues, but not in xylem parenchyma, vascular cambium, or any other cell types of vegetative tissues. The differences observed for the *Sh* promoter expression in the roots of corn and tobacco may be due to differences inherent in the expression of the corn *Sh* promoter in the two species. Alternatively, they may be due to differences in the resolution of the different assay systems used. A direct comparison using the same assay systems or access to *Sh-Gus* transgenic corn would address these differences.

At present, the physiological role(s) of the sucrose synthase enzyme(s) in vegetative tissues of plants is not clear (7). Our observation on cell-type expression of the *Sh* promoter suggests that a physiological role of sucrose synthase activity may be specifically associated with the phloem tissues of plants—e.g., sucrose loading and transport through the phloem cells in vascular plants.

The fact that the *Sh-Gus* gene is effectively expressed in tobacco phloem cells suggests that both the transcriptional and translational apparatus in tobacco phloem are capable of recognizing the signals of corn *Sh* promoter and its intron sequences and can effectively utilize them to dictate the induction and cell-type-specific expression of the *Gus* coding sequences.

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