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Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase

(nitrogen/phloem transport/light regulation/β-glucuronidase/gene expression)

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ABSTRACT **Chloroplast and cytosolic isoforms of glutamine synthetase (GS; EC 6.3.1.2) are encoded by separate nuclear genes in plants. Here we report that the promoters for chloroplast GS2 and cytosolic GS3A of *Pisum sativum* confer nonoverlapping, cell-specific expression patterns on the β-glucuronidase (GUS) reporter gene in transgenic tobacco. The promoter for chloroplast GS2 directs GUS expression within photosynthetic cell types (e.g., palisade parenchymal cells of the leaf blade, chlorenchymal cells of the midrib and stem, and photosynthetic cells of tobacco cotyledons). The promoter for chloroplast GS2 retains the ability to confer light-regulated gene expression in the heterologous transgenic tobacco system in a manner analogous to the light-regulated expression of the cognate gene for chloroplast GS2 in pea. These expression patterns reflect the physiological role of the chloroplast GS2 isoform in the assimilation of ammonia generated by nitrite reduction and photorespiration. In contrast, the promoter for cytosolic GS3A directs expression of GUS specifically within the phloem elements in all organs of mature plants. This phloem-specific expression pattern suggests that the cytosolic GS3A isoenzyme functions to generate glutamine for intercellular nitrogen transport. In germinating seedlings, the intense expression of the cytosolic GS3A-GUS transgene in the vasculature of cotyledons reveals a role for cytosolic GS in the mobilization of seed storage reserves. The distinct, cell-specific patterns of expression conferred by the promoters for chloroplast GS2 and cytosolic GS3A indicate that the corresponding GS isoforms perform separate metabolic functions.**

In higher plants, many steps in nitrogen metabolism occur in multiple subcellular compartments. For example, many amino acid biosynthetic isoenzymes are located in the cytosol as well as in the mitochondria or chloroplasts. The relative function of many amino acid biosynthetic isoenzymes has been difficult to assess due to inadequate fractionation of organelle and cytoplasm components, overlapping activity profiles, and immunological cross-reactivity (1, 2). Consequently, it is unclear whether these isoenzymes carry out redundant or distinct roles in plant metabolism.

The best-studied example of a plant amino acid biosynthetic enzyme shown to occur as multiple isoforms is glutamine synthetase (GS; EC 6.3.1.2) (3). Early biochemical data revealed that GS functions in the assimilation of ammonia generated by numerous plant processes, which include seed germination (4, 5), photorespiration (6, 7), nitrite reduction (8), nitrogen-fixation in root nodules (9, 10), and primary ammonia assimilation from the soil (11). An analysis of the GS genes in several species has revealed a strong correlation of individual GS gene expression with specific aspects of plant development (12–18). Recent sequence analysis of GS cDNAs from *Pisum sativum* and *Phaseolus vulgaris* has shown that chloroplast and cytosolic GS are encoded by separate but similar nuclear genes (12, 13, 19).

In pea, the single nuclear gene for chloroplast GS2 is expressed predominantly in leaves in a light-dependent fashion (13, 17). The role of chloroplast GS2 in the reassimilation of photorespiratory ammonia is supported by the analysis of mutants in barley (7) and is substantiated by gene expression studies in pea (17). For cytosolic GS, molecular studies have revealed the presence of a number of distinct isoforms in several plant species (12, 14, 16, 20). In pea it has been shown that two classes of genes encode homologous but distinct cytosolic GS isoforms (13). One class comprises two nearly identical GS genes [GS3A (also known as GS341) and GS3B (also known as GS132)] whose expression is specifically induced in two organs where large amounts of ammonia are mobilized for plant growth, in cotyledons of germinating seedlings, and in nitrogen-fixing root nodules (18).

Here, we show that the promoters isolated from the nuclear genes for chloroplast GS2 and cytosolic GS3A of pea are able to direct cell-specific and regulated expression of the β-glucuronidase (GUS) reporter gene (21) in transgenic tobacco plants. The nonoverlapping patterns of expression indicate that the chloroplast GS2 and cytosolic GS3A isoforms do not perform redundant functions during plant development. Therefore, in addition to defining salient features of GS gene expression, the results of these transgenic experiments have provided a molecular basis for discriminating the physiological functions of closely related isoenzymes.

MATERIALS AND METHODS

Isolation of GS Genomic Clones. Genomic clones encoding chloroplast or cytosolic GS of pea were isolated from a genomic library of *Pisum sativum* cv. "Sparkle" (Rogers Brothers Seed, Twin Falls, ID) constructed in "Lambda Dash" (Stratagene). Complete sequence analysis of each genomic clone (unpublished data) revealed that the genomic clone for chloroplast GS2 ($GS2^{ct}$) corresponds to the GS185 cDNA (13), whereas the genomic clone for cytosolic GS ($GS3A^{cy}$) corresponds to the GS341 cDNA (12, 18).

Construction of Plasmids and Transformation of Agrobacterium. A 1.5-kilobase (kb) *Eco*RI–*Hin*cII fragment of the 5′ end of the pea nuclear gene for chloroplast GS2 ($GS2^{ct}$) was cloned into pBI101.2 (22) (Clontech) to create the plasmid $pGS2^{ct}$-GUS (Fig. 1*A*). The $pGS2^{ct}$-GUS construct contains the promoter of GS2, ≈65 nucleotides of the 5′ untranslated leader of the GS2 mRNA, and encodes 53 amino acids of the chloroplast transit peptide (13). The $GS3A^{cy}$-GUS fusion was constructed by inserting a 1.01-kb DNA fragment encompassing nucleotide position −903 to a *Bgl* II site at position +107 of the $GS3A^{cy}$

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Abbreviations: GS, glutamine synthetase; GUS, β-glucuronidase.
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gene into the *Bam*HI site upstream of the GUS gene in pBI101 (22). The GS3A^{cy}-GUS fusion gene was released from the plasmid as a *Sal* I–*Eco*RI fragment that was subsequently cloned into the *Xho* I–*Eco*RI sites of pMON505 (23) to create the plasmid pGS3A^{cy}-GUS (Fig. 1*B*). pGS3A^{cy}-GUS contains 88 nucleotides of the 5′ untranslated leader of GS3A mRNA and encodes 6 amino acids of the cytosolic GS protein (12).

Transformation and Growth of Transgenic Tobacco Plants. Binary vectors containing the pGS2^{ct}-GUS or pGS3A^{cy}-GUS constructs were transferred into the disarmed *Agrobacterium* strain LBA4404 or GV311SE, respectively, by triparental mating as described (24). *Nicotiana tabacum* cv. SR1 or *Nicotiana tabacum* cv. Xanthi was transformed by a leaf inoculation procedure (25). Regenerated shoots were selected for growth on medium containing kanamycin (200 μg/ml). Primary transformants were maintained in sterile culture and were also grown to maturity in soil. F_1 seeds were sterilized in 10% sodium hypochlorite and germinated on MS medium containing 3% sucrose, 100 μg of kanamycin per ml, and 500 μg of carbenicillin per ml. Seedlings were grown in culture for 7 days at 26°C in continuous white light (4000 lux).

Determination of GUS Expression. GUS enzyme assays and histochemical staining of mature plants and seedlings were performed as described (22, 26, 27). After incubation with the GUS substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Clontech), cross sections of mature plant organs and whole seedlings were cleared of chlorophyll by incubation with a solution of 5% formaldehyde/5% acetic acid/20% ethanol for 10 min followed by 2-min incubations with 50% and 100% ethanol. Photomicrographs were taken with a Nikon Optiphot microscope using phase-contrast optics.

Plant Growth Conditions for Light-Induction Experiments. Transgenic plants containing the GS2^{ct}-GUS fusion gene were germinated and grown in soil in continuous light for 4 weeks. The plants were transferred to black Lucite boxes within a dark environmental chamber for 4 days. Several fully expanded leaves of each plant were collected in the dark and immediately frozen in liquid nitrogen. The plants were subsequently transferred to continuous light for 24 hr and several fully expanded leaves were collected and frozen for RNA extraction.

Isolation of RNA and RNase Protection Assay. RNA was extracted from leaves of dark-adapted and light-grown transgenic tobacco plants using guanidine thiocyanate as a protein denaturant (28). RNase protection assays were performed using a DNA vector (pJE1005) (J.W.E. and G.M.C., unpublished results) that contained a 1.5-kb *Eco*RI–*Hin*cII fragment of the nuclear gene for chloroplast GS2 (GS2^{ct}) in the plasmid pTZ18U (United States Biochemical). A DNA template encompassing the 5′ end of GS2^{ct} was generated by *Hin*dIII digestion of pJE1005 and was used to create a radioactive, antisense RNA probe *in vitro* using T7 RNA polymerase (29). Fifty micrograms of total RNA from transgenic tobacco plants was hybridized to an excess of the antisense RNA probe overnight in 80% formamide/60 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA at 60°C. RNase T2 digestions were performed in a volume of 390 μl containing 50 mM NaOAc (pH 5.0), 100 mM NaCl, 2 mM EDTA, and 60 units of RNase T2 per ml (Bethesda Research Laboratories) (30). Digestion products were separated on an 8% acrylamide/7 M urea gel and exposed to x-ray film at −80°C.

RESULTS

Construction of GS-GUS Reporter Gene Fusions and Quantification of GUS Activity in Transgenic Plants. The genomic clone pGS2^{ct} corresponds to the single nuclear gene for chloroplast GS2 (13) and the genomic clone pGS3A^{cy} encodes the predominant mRNA (GS341) for cytosolic GS in pea (18). Promoter elements from GS2^{ct} and GS3A^{cy} were subcloned as translational fusions to the GUS reporter gene to create pGS2^{ct}-GUS and pGS3A^{cy}-GUS (Fig. 1) and were introduced into *Nicotiana tabacum* (see *Materials and Methods*).

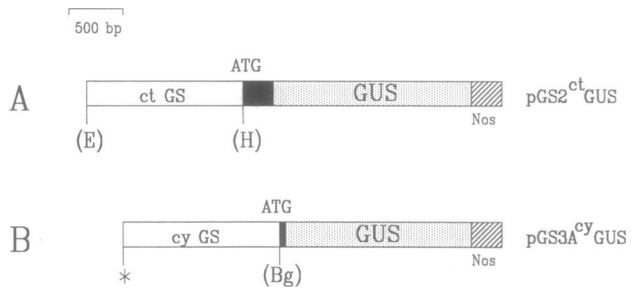

FIG. 1. GS-GUS translational fusions. (*A*) pGS2^{ct}-GUS contains 1.5 kb from the 5′ end of the gene for chloroplast GS2 in a translation fusion with the GUS gene of pBI101.2. (*B*) pGS3A^{cy}-GUS contains 1.01 kb from the 5′ end of the gene for cytosolic GS3A in a translational fusion with the GUS gene of pBI101. A 3′ poly(A) region from the nopaline synthase (Nos) gene is present in both GS-GUS constructs and is denoted with diagonal stripes. The white areas represent the 5′ noncoding region of each GS gene, the solid black areas depict GS coding regions, and the dotted area marks the GUS coding region. Restriction sites: E = *Eco*RI, H = *Hin*cII, Bg = *Bgl* II. The asterisk indicates position −903. Restriction sites in parentheses indicate original sites in plant genes that were destroyed in plasmid construction. bp, Base pairs.

GUS enzyme activity was measured in soluble protein extracts of leaves of 19 individual transgenic plants by a fluorimetric assay (22, 26) (Table 1). GUS activity in leaves of primary transgenic plants containing the pGS2^{ct}-GUS construct averaged 46,984 pmol of methylumbelliferone per mg of protein per min, whereas GUS activity in the leaves of transgenic plants containing the pGS3A^{cy}-GUS chimeric construct was ≈17-fold lower. Southern blot analysis revealed that each transformed plant contained one or two copies of the GS-GUS transgene (data not shown).

The Promoter for Chloroplast GS2 Directs GUS Expression Specifically in Photosynthetic Cell Types. *In situ* stained sections of the pGS2^{ct}-GUS transgenic plants reveal that the promoter for chloroplast GS2 directs high-level GUS expression in leaves, specifically in the leaf blade (Fig. 2*A*). The most intense GUS staining occurs in the palisade parenchymal cells of the leaf blade, which contain a large number of chloroplasts (Fig. 2*B*). In a cross section of the leaf midrib, pGS2^{ct}-GUS activity is detected in only two photosynthetic cell layers (collenchyma and chlorenchyma) and not in the

Table 1. GUS activity in leaves of transgenic tobacco plants

Chloroplast GS2^{ct}-GUS transformant		Cytosolic GS3A^{cy}-GUS transformant	
Construct	No.	Construct	No.
pGS2^{ct}-GUS-1	13,070	pGS3A^{cy}-GUS-1	2183
pGS2^{ct}-GUS-2	22,374	pGS3A^{cy}-GUS-2	9429
pGS2^{ct}-GUS-3	53,155	pGS3A^{cy}-GUS-3	1940
pGS2^{ct}-GUS-4	67,300	pGS3A^{cy}-GUS-4	372
pGS2^{ct}-GUS-5	60,373	pGS3A^{cy}-GUS-5	648
pGS2^{ct}-GUS-6	32,918	pGS3A^{cy}-GUS-6	2453
pGS2^{ct}-GUS-7	43,084	pGS3A^{cy}-GUS-7	1740
pGS2^{ct}-GUS-8	53,886		
pGS2^{ct}-GUS-9	40,802		
pGS2^{ct}-GUS-10	95,435		
pGS2^{ct}-GUS-11	43,568		
pGS2^{ct}-GUS-12	37,840		
Average	46,984		2681

GUS activity is expressed as pmol of methylumbelliferone per mg of protein per min.

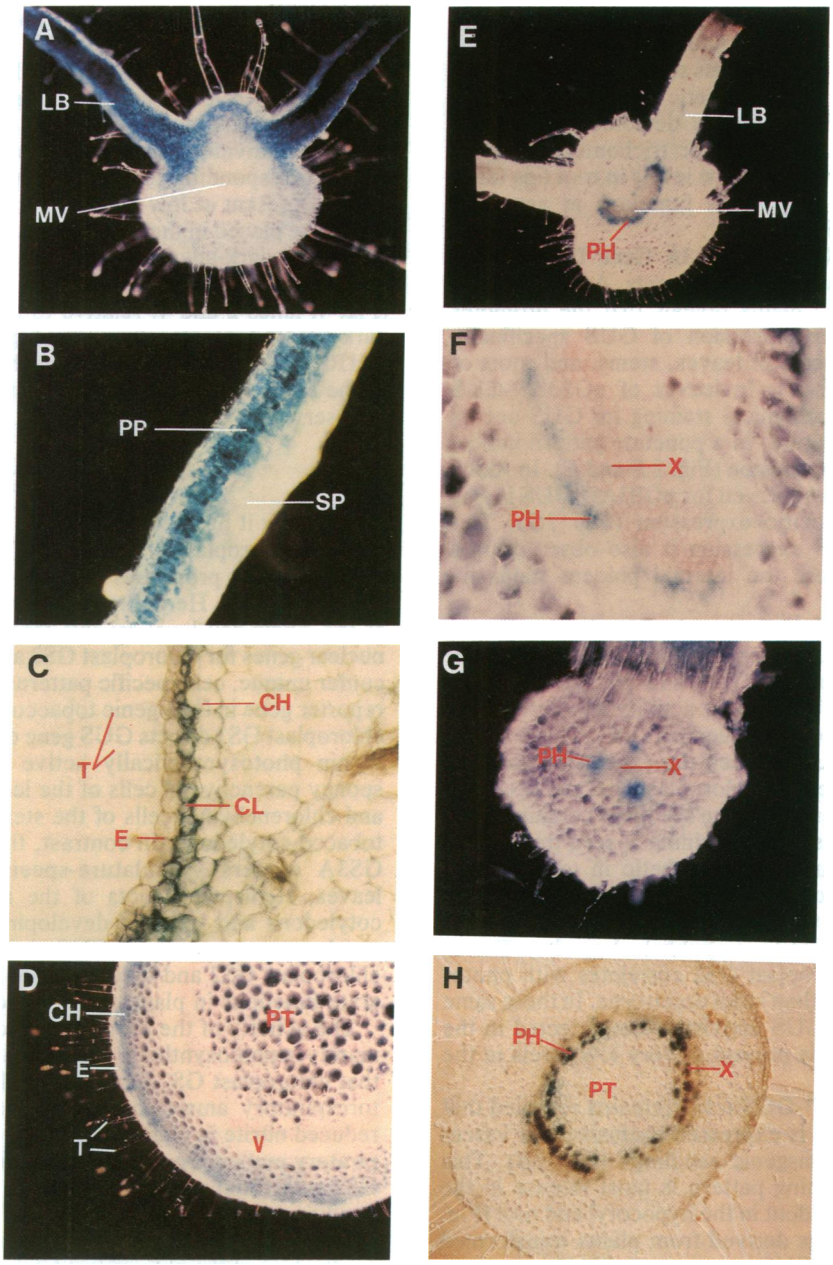

FIG. 2. Histochemical localization of GUS activity in cross sections of mature transgenic tobacco plants. (*A–D*) Sections from pGS2^{ct}-GUS transformants. (*A*) Leaf cross section. (*B*) Leaf blade cross section. (*C*) Leaf midrib cross section. (*D*) Stem cross section. (*E–H*) Sections from pGS3A^{cy}-GUS transformants. (*E*) Leaf cross section. (*F*) Leaf midrib cross section. (*G*) Root cross section. (*H*) Stem cross section. CH, chlorenchyma; CL, collenchyma; E, epidermis; LB, leaf blade; MV, midvein; PH, phloem; PP, palisade parenchyma; PT, pith parenchyma; R, root; SP, spongy parenchyma; T, trichome; V, vasculature; X, xylem.

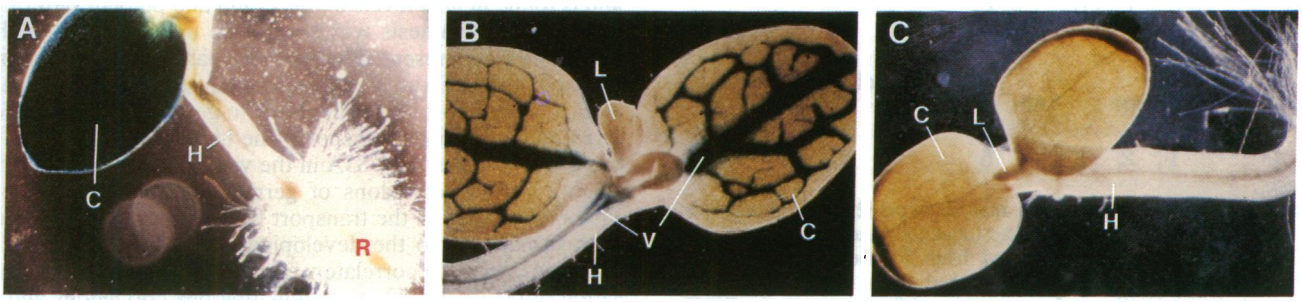

FIG. 3. Histochemical localization of GUS activity in whole mounts of 7-day-old transgenic tobacco seedlings. (*A*) pGS2^{ct}-GUS transformant. (*B*) pGS3A^{cy}-GUS transformant. (*C*) Control, pBI101 transformant. C, cotyledon; H, hypocotyl; L, leaf; R, root; V, vasculature.

adjacent epidermal cell layer (Fig. 2*C*). There is no GUS expression in the central vascular bundle of the midvein in the pGS2^{ct}-GUS plants (Fig. 2*A*). In cross sections of stem, GUS activity is detected in the photosynthetic chlorenchymal cells (Fig. 2*D*), and no GUS staining is detected in the pith parenchymal, vascular, epidermal, or trichome cells (Fig. 2*D*). pGS2^{ct}-GUS is expressed at low levels in root tips (data not shown), where GS in plastids functions in ammonia assimilation from the soil (8).

The Promoter for a Cytosolic GS3A Gene Directs GUS Expression Exclusively in Vascular Elements. Analysis of the pGS3A^{cy}-GUS transgenic plants reveals that the promoter for cytosolic GS directs expression of GUS specifically within the vascular elements of leaves, stems, and roots of mature plants (Fig. 2 *E–H*). In leaves of pGS3A^{cy}-GUS transgenic tobacco, histochemical staining for GUS occurs exclusively in the vasculature, in a punctate pattern indicative of phloem-specific expression (Fig. 2 *E* and *F*). In roots, the triarc staining pattern observed for pGS3A^{cy}-GUS is also indicative of phloem-specific expression (Fig. 2*G*). This punctate pattern of GUS expression is also observed in a stem cross section, where the internal phloem stains intensely (Fig. 2*H*).

Expression of the GS-GUS Fusions in Germinating Transgenic Tobacco Seedlings. To examine the organ and cell-specific expression of pGS2^{ct}-GUS and pGS3A^{cy}-GUS in germinating seedlings, GUS enzyme activity was detected *in situ* in whole mounts of tobacco seedlings (27). This analysis reveals a striking contrast between the expression patterns conferred by the promoters for chloroplast GS2 and cytosolic GS3A (Fig. 3). In transgenic tobacco seedlings containing the pGS2^{ct}-GUS gene, intense GUS staining is seen throughout the cotyledons, which are photosynthetic in tobacco (31) (Fig. 3*A*). In pea cotyledons, which are nonphotosynthetic (32), there is low-level expression of the mRNA for chloroplast GS2 (J.W.E. and G.M.C., unpublished results). Therefore, expression of chloroplast GS2 correlates with photosynthetic capacity rather than strict organ type. In these same pGS2^{ct}-GUS seedlings, GUS activity is not detected in the hypocotyl (Fig. 3*A*) and is present at very low levels in the root tips (Fig. 3*A*).

In situ stained pGS3A^{cy}-GUS transformants revealed that the cytosolic GS3A gene is expressed exclusively in vasculature of developing transgenic seedlings (Fig. 3*B*). This vasculature-specific staining pattern is most intense in the cotyledons and is also evident in the hypocotyl and root (Fig. 3*B*). Control, F_1 seedlings derived from plants transformed with a "promoterless" GUS construct (pBI101) show no detectable GUS activity in histochemical assays (Fig. 3*C*).

The Chloroplast GS2 Promoter Confers Light-Regulated Expression on the GUS Reporter Gene. Previous results have demonstrated that light induces the accumulation of the mRNA for chloroplast GS2 in mature pea plants and in etiolated seedlings (17). To determine whether the promoter for chloroplast GS2 is responsible for the light-induced accumulation of the mRNA for chloroplast GS2, levels of GS2^{ct}-GUS RNA present in transgenic plants grown in the light or dark were measured in a RNase protection assay (Fig. 4). In two individual transgenic plants assayed, the amount of RNA corresponding to the chloroplast GS2-GUS chimeric RNA is present at low levels when the mature, light-grown plants are placed in the dark for 4 days (Fig. 4, lanes 1 and 3). When the dark-adapted plants are returned to continuous light for 24 hr, the GS2^{ct}-GUS mRNA accumulated ≈8-fold (Fig. 4, lanes 2 and 4) relative to the levels detected in the dark-adapted plants. In control plants transformed with pBI101 and grown in continuous light, no cross-hybridization of the RNA probe with the endogenous tobacco GS mRNA is observed (Fig. 4, lane 5).

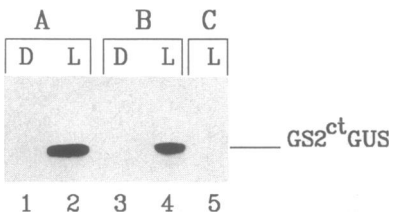

FIG. 4. RNase T2 protection analysis of pGS2^{ct}-GUS transcripts in light- vs. dark-grown transgenic tobacco. Autoradiograph of the 162-nucleotide fragment protected from RNase T2 digestion in hybridizations containing 50 μg of total RNA isolated from two separate pGS2^{ct}-GUS transformants (*A* and *B*) and a control, pBI101 transformant (*C*) that were dark-adapted for 4 days (lanes 1 and 3) and subsequently grown in continuous light for 24 hr (lanes 2, 4, and 5).

DISCUSSION

Historically it has been difficult to assess the relative functions of chloroplast and cytosolic GS due to similarities in their physical properties as well as their immunological cross-reactivity. Here, the ability to localize gene expression at the single-cell level reveals that the promoters from the nuclear genes for chloroplast GS2 and cytosolic GS3A of pea confer unique, cell-specific patterns of expression on a GUS reporter gene in transgenic tobacco plants. The promoter for chloroplast GS2 directs GUS gene expression predominantly within photosynthetically active cells, the palisade and spongy parenchymal cells of the leaf blade, in collenchymal and chlorenchymal cells of the stem, and in photosynthetic tobacco cotyledons. In contrast, the promoter for cytosolic GS3A confers vasculature-specific GUS expression in leaves, stems, and roots of the mature plant and in the cotyledons and roots of developing seedlings. These nonoverlapping patterns of GUS expression signify that the chloroplast GS2 and cytosolic GS3A isoforms perform separate functions in plant nitrogen metabolism.

The activity of the promoter for chloroplast GS2 predominantly in photosynthetic cell types is consistent with findings that chloroplast GS2 functions in the reassimilation of photorespiratory ammonia (7, 17) and in the assimilation of reduced nitrite in plastids (8). Previous analysis of photorespiratory mutants revealed that plants that lacked chloroplast GS2 were nonviable when grown under photorespiratory conditions even though they contained normal levels of cytosolic GS (7). The inability of cytosolic GS to compensate for the loss of the chloroplast GS2 activity in photosynthetic cells of mutant plants (7) may be explained by the fact that cytosolic GS and chloroplast GS2 are expressed in separate cell types, as demonstrated here.

The unforeseen finding of this transgenic analysis was the confinement of cytosolic GS3A gene expression exclusively to the vascular elements. Although glutamine serves as a major compound for intercellular nitrogen transport in higher plants, and is found in high levels in the xylem and phloem saps (33), its source of synthesis was heretofore unknown. From the transgenic data presented here, it is apparent that at least one cytosolic GS isoform is expressed exclusively in the phloem elements and most likely functions to generate glutamine for intercellular nitrogen transport. The high-level expression of the gene for cytosolic GS3A in the vasculature is particularly intense in the cotyledons of germinating seedlings, where glutamine serves as the transport form of nitrogen from seed storage reserves to the developing plant. These findings in transgenic tobacco correlate well with the abundant accumulation of GS3A mRNA in germinating pea cotyledons and in nitrogen-fixing root nodules (12, 18). Since expression of the pea GS3A gene in tobacco cotyledons is confined to the vasculature, it will be of interest to determine whether induced

expression of this cytosolic GS isoform in pea nodules (18) is also confined to the vasculature of this organ. Recently, it has been shown that promoters for two cytosolic GS genes of *Phaseolus vulgaris* can direct expression of GUS in transgenic *Lotus corniculatus* nodules and that one of these promoters is active in vascular and cortical cells of the nodule (15).

The amount of GUS activity produced in transgenic plants containing pGS2^{ct}-GUS is comparable to that reported for other "strong" promoters such as that for the cauliflower mosaic virus 35S protein (27) and patatin (34). The quantification of GUS activity detected in whole leaf extracts of plants revealed that plants containing the chloroplast GS2^{ct}-GUS transgene contained, on average, 17 times more GUS activity than plants containing the pGS3A^{cy}-GUS construct. However, because the expression of each of the GS-GUS constructs is confined to distinct cell types that comprise different fractions of the total leaf cell population, the GUS activity in whole leaf extracts cannot be regarded as a measure of absolute promoter strength.

The light-induced accumulation of the chimeric GS2^{ct}-GUS mRNA reveals that the promoter for GS2^{ct} contains a cis-acting DNA element involved in light regulation. Since previous experiments have demonstrated that phytochrome is partially responsible for the light induction of the mRNA for chloroplast GS2 (13, 17), studies of the promoter for GS2^{ct} should contribute to the understanding of phytochrome-mediated gene expression. It is noteworthy that plastid GS2 is also present in nonphotosynthetic cell types such as etiolated leaves (35) and roots (8). This is corroborated by the low level of GUS expression in roots of pGS2^{ct}-GUS transgenic plants (data not shown). Therefore, the regulation of expression of the GS2^{ct} gene is likely to differ from that of other light-regulated genes whose products function solely in photosynthesis (e.g., ribulose 1,5 bisphosphate carboxylase, chlorophyll a/b binding protein).

The unique expression patterns conferred upon the GUS reporter gene by the promoters for chloroplast GS2 and cytosolic GS3A and the light-induced accumulation of pGS2^{ct}-GUS RNA are most likely due to the transcriptional regulation of these transgenes. However, because the GS-GUS fusions contain the 5′ noncoding leader of the GS mRNAs and a small portion of the GS coding regions, it is possible that posttranscriptional events (e.g., RNA stability, translational regulation, and subcellular compartmentalization) also contribute to the observed differences in transgene expression. Future experiments directed at characterizing the specific cis-acting regulatory regions of the GS genes should distinguish between these possibilities.

In addition to elucidating the individual roles of the GS isoforms in plant nitrogen metabolism, the transgenic studies presented here describe plant promoters that may be used to direct cell-specific expression of foreign genes in plants. In particular, a promoter that confers specific expression of foreign genes in phloem cells has potential application in generating resistance to pathogens transmitted within the phloem (36). Finally, since GS is the target of several herbicides (37), the expression studies presented here indicate that it may be necessary to express herbicide-resistant forms of GS in photosynthetic and vascular cell types in order to confer resistance to GS inhibitors.

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