Tissue-Specific and Developmental Pattern of Expression of the Rice *sps1* Gene[1]

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Sucrose-phosphate synthase (SPS) is one of the key regulatory enzymes in carbon assimilation and partitioning in plants. SPS plays a central role in the production of sucrose in photosynthetic cells and in the conversion of starch or fatty acids into sucrose in germinating seeds. To explore the mechanisms that regulate the tissue-specific and developmental distribution of SPS, the expression pattern of rice (*Oryza sativa*) *sps1* (GenBank accession no. U33175) was examined by in situ reverse transcriptase-polymerase chain reaction and the expression directed by the *sps1* promoter using the β-glucuronidase reporter gene. It was found that the expression of the rice *sps1* gene is limited to mesophyll cells in leaves, the scutellum of germinating seedlings, and pollen of immature inflorescences. During leaf development, the *sps1* promoter directs a basipetal pattern of expression that coincides with the distribution of SPS activity during the leaf sink-to-source transition. It was also found that during the vegetative part of the growth cycle, SPS expression and enzymatic activity are highest in the youngest fully expanded leaf. Additionally, it was observed that the expression of the *sps1* promoter is regulated by light and dependent on plastid development in photosynthetic tissues, whereas expression in scutellum is independent of both light and plastid development.

Suc is the main compound used by most plant species to translocate photoassimilates from the leaves to non-photosynthetic tissues, probably due to its high solubility, low reactivity, and energy storage capacity (Akazawa and Okamoto, 1980; Giaquinta, 1980). This carbohydrate is synthesized either from chloroplast carbon assimilation products or from starch accumulated in storage organs. After its biosynthesis in source tissues, Suc moves by a combination of symplasmic and apoplasmic pathways to the phloem where it is loaded by a proton-driven symporter (Riesmeier et al., 1994). Once in the phloem, Suc is translocated by a mass flow to sink tissues. In sink organs, Suc is cleaved by Suc synthase (SS; EC 2.4.1.13) to produce UDP-Glc and Fru, or it is hydrolyzed by invertase (EC 3.2.1.26) to yield Glc and Fru (Giaquinta, 1983), which are then used as energetic and structural sources for multiple biosynthetic pathways.

Suc is synthesized by the coupled action of Suc-P synthase (SPS; EC 2.3.1.14) and Suc-P phosphatase (SPP; EC 3.1.3.00). Transfer of the glucosyl moiety from UDP-Glc to Fru-6-P is catalyzed by SPS, and the Suc-6-P obtained is dephosphorylated by SPP yielding Suc as the final product. Among the enzymes involved in Suc biosynthesis from triose-P in photosynthetic tissues, SPS and Fru-1,6-bisphosphatase have been suggested as the major rate-limiting steps (Kerr and Huber, 1987; Stitt, 1989; Neuhaus et al., 1990).

Changes in leaf SPS activity are reflected in the content of Suc in leaves (Köhler et al., 1988; Lunn and Furbank, 1997; Grof et al., 1998). SPS activity has not only been found to correlate with the availability of Suc for distribution to sink tissues (Stitt et al., 1988; Walker and Huber, 1989; Neuhaus et al., 1990; Prioul et al., 1990; Reimholz et al., 1997) but also with the accumulation of this disaccharide as a response to environmental stresses, including water deficit and low temperature (Quick et al., 1989; Tognetti et al., 1989; Guy et al., 1992; Ingram et al., 1997; Toroser and Huber, 1997; Escobar-Gutiérrez et al., 1998).

The availability of substrates for Suc biosynthesis in leaves depends on their photosynthetic capacity.

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During development, leaves undergo a transition from sink (net photoassimilate importer) to source (net photoassimilate exporter) status, which has been shown to initiate at the tip of young leaves and to follow a basipetal pattern toward the end of the leaf base (Turgeon, 1989; Roberts et al., 1997). In this regard it is interesting to note that in mature maize leaves SPS accumulation shows a basipetal gradient reflecting the sink-to-source transition (Bruneau et al., 1991). In addition to its role in Suc biosynthesis in leaves, SPS is also involved in Suc accumulation in ripening fruits, in sprouting tubers, and in germinating seeds (Nomura et al., 1969; Hubbard et al., 1990; Castrillo et al., 1992; Reimholz et al., 1994; Cordenunsi and Lajolo, 1995; Reimholz et al., 1997).

In agreement with its proposed functions, SPS activity has been detected in mesophyll cells (Furbank et al., 1985) and the scutellum of germinating seeds (Nomura et al., 1969; Nomura and Akazawa, 1973). The level of SPS activity in source tissues appears to be mainly regulated, at the enzyme level, by allosteric (Doehlert and Huber, 1984) and covalent (Huber and Huber, 1990; Huber and Huber, 1991; Toroser and Huber, 1997) modifications. In contrast with this, the developmental and tissue-specific distribution of SPS has been suggested to be regulated at the transcriptional level (Harn et al., 1993; Klein et al., 1993; Cheng et al., 1996; Valdez-Alarcón et al., 1996).

Plant cDNA clones encoding SPS have been isolated from maize (Worrell et al., 1991), spinach (Klein et al., 1993; Sonnewald et al., 1993), potato (EMBL accession no. S34172), sugar beet (Hesse et al., 1995), *Citrus unshiu* (Komatsu et al., 1996), faba bean (Heim et al., 1996), banana (do Nascimento et al., 1997), *Craterostigma plantagineum* (Ingram et al., 1997), sugarcane (Sugiharto et al., 1997), and kiwi fruit (Langerkämper et al., 1998). However, to date, genomic sequences have only been described for rice (*Oryza sativa*) *sps1* (GenBank accession no. U33175) gene (Sakamoto et al., 1995; Valdez-Alarcón et al., 1996).

As an initial step to elucidate the mechanisms that regulate the tissue-specific and developmental distribution of SPS, we set out to detect *sps1* mRNA by in situ reverse transcriptase (RT)-PCR amplification and to analyze the pattern of expression directed by the rice *sps1* promoter in transgenic rice plants using β-glucuronidase (*GUS*) as a reporter gene.

RESULTS

Rice *sps1* Promoter Contains Several Putative cis-Acting Regulatory Elements

We have previously reported the isolation and characterization of the rice *sps1* genomic clone and mapped its transcription initiation site (Valdez-Alarcón et al., 1996). In Figure 1A, the nucleotide sequence of a 2,196-bp *sps1* promoter fragment is presented. Computer and visual analysis of the *sps1* promoter sequence allowed us to identify a number of DNA motifs potentially involved in the transcriptional regulation of this gene. Putative regulatory elements of two major classes were found to be present in the *sps1* promoter: (a) DNA motifs similar to light-responsive elements (LREs), which are involved in light- and tissue-specific regulation of photosynthesis related genes, such as the I-box (TGGTGNNYAAY-GATAAGG; Argüello-Astorga and Herrera-Estrella, 1996), G-box (CACGTG; Giuliano et al., 1988), as well as AT-1 like binding sites (AATATTTTTATT; Bansal et al., 1992) and (b) DNA motifs closely related to gibberellin-response elements (GARE) present in α-amylase genes such as the Amybox 1 (TAACARA), the O2S box (TATCCAY), and the pyrimidine box (YCTTTTY) (Huang et al., 1990). In addition, a 16-bp sequence (RTGCAATRYWWRAT) present in the pro-

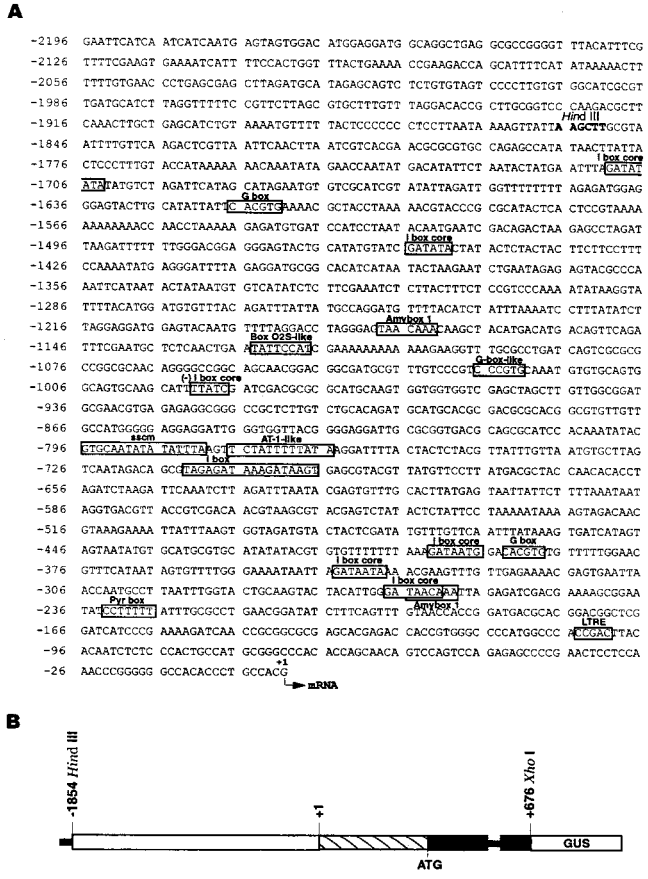

Figure 1. *sps-gus1* gene construction. A, Nucleotide sequence of the *sps1* promoter region. Putative regulatory cis-acting elements are indicated. The transcription initiation site is indicated with +1. The *Hin*dIII restriction site used to obtain the *sps-gus1* construct is indicated in bold letters. B, Structural mapping of the regulatory region of the s*ps-gus1* construct. Numbers indicate position relative to the transcription start site. Promoter region is indicated by an white box, leader region by a hatched box, and exons as black boxes. References for the described sequences are: G-box (Giuliano et al., 1988); AT-1 box (Bansal et al., 1992); I-box (Giuliano et al., 1988); I box core (Gidoni et al., 1989); gibberellin response elements (Huang et al., 1990); and low temperature response elements (Baker et al., 1994). SSCM stands for SPS-SS conserved motif.

moter of two rice SS genes (*Rsus1* [Wang et al., 1992] and *Rsus2* [Yu et al., 1992]) and the core of a low temperature responsive element (CCGAC, Baker et al., 1994) were also found in the *sps1* promoter.

sps1 Expression in Leaves

To examine the tissue-specific expression of *sps1* in leaves, in situ RT-PCR reactions, using Oregon Green 488-5-dUTP as fluorescent label, where carried out on fresh tissue sections of rice leaves and germinating seeds. This technique has proven to yield high-resolution of specific mRNA amplification signals on several plant tissues (Ruiz-Medrano et al., 1999; Xoconostle-Cázares et al., 1999).

In leaves, *sps1* amplification products were clearly detected in mesophyll cells and undetectable in vascular tissues (Fig. 2A). The presence of *sps1* tran-

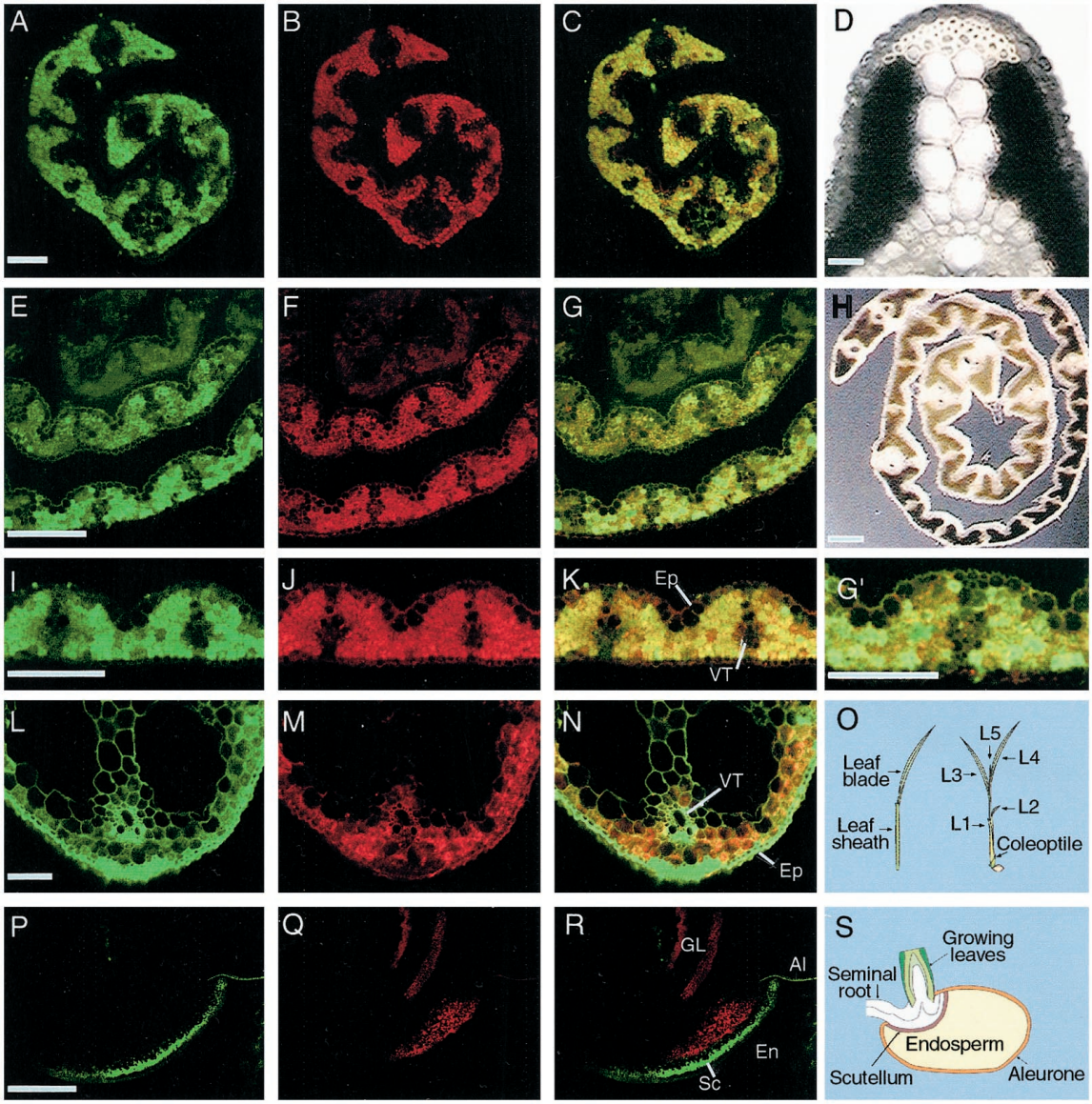

Figure 2. Histological localization of *sps1* expression in rice. Leaf and seed samples were sectioned and subjected to in situ RT-PCR. After the reaction, fluorescent signals were detected by confocal laser scanning microscope. Samples were processed to detect *sps1* (A, E, and P), *rbcS* (I), and actin (L) transcripts. Images are as follows: apex (A–C) or base (E–G) of a sheathed rice leaf; higher magnification of G (G′); leaf blade (I–K), leaf sheath (L–N), and 3-d-old germinating seeds (P–R). A, E, I, L, and P, Green fluorescent signals of mRNA amplification; B, F, J, M, and Q, chlorophyll autofluorescence from the same field of view presented in A, E, I, L, and P, respectively. C, G, K, N, and R, Overlap of green and red fluorescences from the same field of view presented in A, C, I, L, and P, respectively. D, Starch localization in a leaf blade midrib. H, Starch localization in the base of a sheathed leaf. Schematic representation of rice leaves (O) and a germinating seed (S). The scale bars represent 20 μm (D), 50 μm (A–C, E–H, and P–R), 100 μm (I–K and G′), and 500 μm (L–N). En, Endosperm; Ep, epidermis; GL, growing leaves; Sc, scutellum; VT, vascular tissue.

script, only in photosynthetically active cells, was confirmed when the red chlorophyll autofluorescence (Fig. 2B) was superimposed with the green signal produced by the in situ RT-PCR reaction (Fig. 2C). To compare the *sps1* expression with the source capacity of the tissue, starch content was determined in expanded leaves by staining with an iodine solution. Starch was present only in mesophyll cells (Fig. 2D). These results indicate that, in leaves, *sps1* is specifically expressed in cells where photosynthates are available and Suc biosynthesis is taking place.

When *sps1* transcript was amplified in sheathed leaves by in situ RT-PCR, a gradient of fluorescence was observed in the leaf base, being higher in the outermost zone (and therefore the more exposed to light) (Fig. 2E). Chlorophyll autofluorescence was found to present a similar pattern of distribution as that shown by *sps1* transcript amplification products (Figs. 2, F, G, and G′). To compare *sps1* expression with the source capacity of the tissue, starch content was determined in the base of leaves that were at the same developmental stage. Starch localization suggested a difference in the source capacity of this tissue in accordance to the *sps1* transcript amplification and chlorophyll detection (Fig. 2H).

To confirm the specificity of the in situ RT-PCR reactions, different control reactions were carried out. When primers specific for the rice *rbcS* transcript where used, in situ RT-PCR showed strong signals of amplification localized in mesophyll cells (Fig. 2, I–K), where *rbcS* expression has been previously reported (Kyozuka et al., 1993). When primers designed for amplification of actin mRNA sequences were used, green fluorescent signals were observed in all leaf cells (Fig. 2, L–N), including vascular tissues and epidermis. These results confirm that the signals produced by the in situ RT-PCR technique we used faithfully represent the in vivo distribution of mRNAs.

To localize seed *sps1* expression during germination, tissue sections of 3-d-old rice seedlings were used for in situ RT-PCR reactions. Amplification signals of *sps1* mRNA were only observed in the seed scutellum (Fig. 2, P and R). In contrast, chlorophyll was observed only in emerging leaves but not in the scutellum (Fig. 2, Q and R). The signal observed in the aleuron layer represent unspecific binding of the fluorescent label, since it was observed in control reaction in which the PCR primers were omitted (data not shown).

Expression of the *sps-gus1* Chimeric Gene in Transgenic Rice Plants

To assess the contribution of the 5′ region of *sps1* gene in the distribution of the SPS mRNA, a 2.5-kb fragment, containing 1.8 kb of 5′ flanking sequences, the 5′-untranslated region, the first exon, the first intron, and part of the second exon, was transcriptionally fused to the reporter gene, GUS (*sps-gus1* chimeric gene, Fig. 1B). This chimeric gene was used to produce transgenic rice plants. Twenty transgenic rice lines were obtained in which the presence of the *sps-gus1* chimeric gene was confirmed by Southern-blot analysis (data not shown). GUS enzymatic activity and in situ localization experiments were carried out on 10 independent transgenic rice lines. Because the *sps-gus1* pattern of expression was very similar for all the lines tested, the results presented in this study are based on the analysis of one representative line (*sps-gus1-r1*).

When examining mature transgenic rice leaves, the expression of *sps-gus1* gene was limited to the mesophyll cells of leaf blades (Fig. 3A) and leaf sheaths (Fig. 3, C and D). A closer inspection showed the absence of GUS activity in vascular tissues of leaf blades (Fig. 3B) and leaf sheaths (Fig. 3E).

sps-gus1 expression was also observed in the mesophyll cells of sheathed leaves, in which a gradient of GUS activity was detected, being higher in the outermost and lower in the innermost zone of the leaf base (Fig. 3F). These results showed a cell-specific expression pattern of the *sps-gus1* gene similar to the *sps1* expression pattern observed in leaves by in situ RT-PCR.

Tissue-specific and developmental expression of *sps-gus1* was also examined in germinating transgenic rice seedlings. Whereas no GUS activity was detected in dry seeds (Fig. 3I), the chimeric gene showed a localized expression in the scutellum of 2-d-old seedlings (Fig. 3J). *sps-gus1* expression increased substantially at day 4 (Fig. 3K), time at which GUS activity was observed in the scutellum and base of the growing leaves. These results show that the *sps1* promoter fragment present in the gene *sps-gus1* gene is capable of directing a tissue-specific and developmental pattern of expression totally consistent with the *sps1* mRNA distribution as detected by in situ RT-PCR.

It has been observed that the level of SPS in expanded corn leaves is not constant, being higher in the dark-green upper part of the leaf and almost undetectable at its base. This gradient of SPS coincides with the distribution of phospho*enol*pyruvate carboxylase, suggesting that the level of SPS varies depending on the readiness of the tissue for CO_2 fixation (Bruneau et al., 1991). To determine whether the distribution of SPS in rice leaves is similar to that reported for maize and correlates with the expression pattern directed by *sps-gus1*, SPS and GUS activity in different regions of a fully expanded leaf were analyzed. A gradient of SPS activity and GUS expression along fully expanded leaves of *sps-gus1* plants was observed, decreasing from the apex toward the lower leaf sheath (Fig. 4, A and B, respectively).

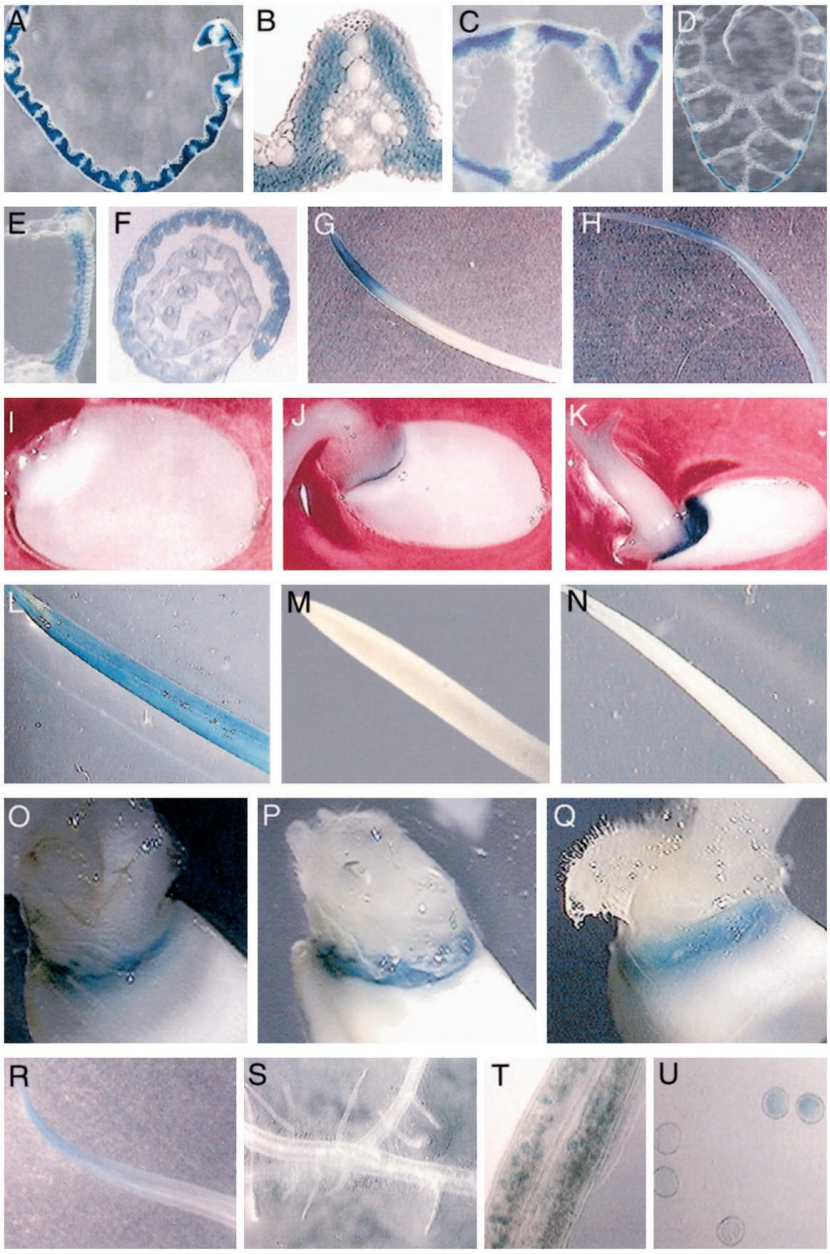

Figure 3. Tissue-specific expression of *sps-gus1* in rice plants. Samples were processed for the localization of GUS activity as described in "Materials and Methods." A, Leaf blade; B, higher magnification of a leaf blade midrib; C, upper leaf sheath; D, lower leaf sheath; E, higher magnification of D; F, base of an sheathed leaf; G and H, primary leaves of 5-d-old (G) and 10-d-old (H) seedlings grown under photoperiod. I to K, Half sections of seedlings during germination d 0 (I), d 3 (J), and d 4 (K). Primary leaves (L–N) and seeds (O–Q) of 7-d-old seedlings grown under photoperiod (L and O), darkness (M and P), and photoperiod (N and Q) and in presence of NF. R, Primary leaf of a 10-d-old plant grown in darkness. S, Roots; T, anther of an immature inflorescence; U, pollen from the immature inflorescence.

Expression during Sink-to-Source Transition in Leaves

To determine whether *sps1* directs a regulated expression during leaf development, the expression directed by *sps-gus1* was analyzed in young emerging leaves and compared with that present in mature, fully expanded leaves. It was observed that in young emerging leaves, GUS activity was only detected in the leaf apex (Fig. 3G). In contrast GUS activity was detected along the entire leaf blade in fully mature leaves (Fig. 3H). These results indicate that the expression of *sps1* is regulated during leaf development, following the pattern of sink-to-source transition as it has been previously reported (Turgeon, 1989; Roberts et al., 1997).

sps-gus1 Expression during Plant Development

In grasses, immature leaves are net importers of photoassimilates and have very high invertase and SS activities (Giaquinta, 1978). In contrast, mature leaves export Suc and support the growth of developing leaves (Langer, 1979). As a consequence, older leaves normally have higher rates of Suc biosynthesis compared with younger leaves. To determine whether *sps1* expression is regulated in accordance with this observation, SPS and GUS activity was measured in leaves at different developmental stages using 12-d-old rice plants. Higher levels of activity of both SPS and GUS were found in expanded leaves when compared with sheathed leaves (Fig. 5, A and B). It was

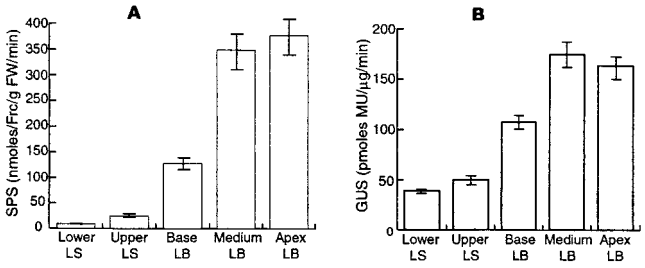

Figure 4. SPS activity and expression pattern along a mature rice leaf. Fully expanded leaves of *sps-gus1* transgenic rice plants were harvested at noon and transversally dissected into five sections where SPS (A) and GUS (B) activity was measured. LS, Leaf sheath; LB, leaf blade.

also observed that the youngest, fully expanded leaf exhibited the highest level of SPS and GUS activity. Similar results were obtained from experiments conducted with eight-leaf greenhouse-grown rice plants, in which the youngest leaf was still sheathed (data not shown).

The Expression of *sps-gus1* in Leaves Is Regulated by Light

The presence of putative LREs in the *sps1* gene promoter suggests that light might play a role in the transcriptional regulation of this gene. To evaluate whether the level of SPS varies depending upon the light regime in which rice plants are grown, SPS activity was determined in leaf tissue of 7-d-old plants grown under continuous darkness or under a 16-h-light/8-h-dark regime. It was observed that SPS activity was 65% higher in light-grown plants compared with those maintained in darkness (Table I).

To examine whether the *sps1* 5′ region directs a light-modulated expression, the level of GUS activity present in the leaves of transgenic *sps-gus1* plants grown under the same conditions as in the previous experiment, was determined. When plants were examined by histochemical GUS assays, no activity was detected in the primary leaves of dark-grown plants

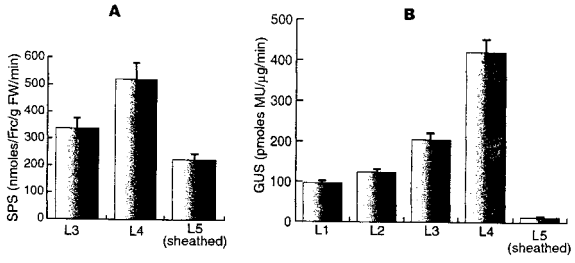

Figure 5. SPS and GUS activity in leaves of 12-d-old plants. SPS (A) and GUS (B) activity in *sps-gus1* transgenic rice leaves. Leaves were separated and analyzed independently by leaf number. L1, L2, L3, L4, and L5 are abbreviations for leaves numbered 1 to 5, respectively, where leaf number 1 corresponds to the oldest leaf, number 4 to the youngest fully expanded leaf, and number 5 to the emerging folded leaf.

Table I. *Effect of light and NF on SPS and GUS activity during leaf development*

SPS and GUS activity was measured in leaf tissue of 7-d-old *sps-gus1* transgenic rice plants harvested at noon. Plants were grown in darkness or under 16-h-light/8-h-dark regime and in the presence or absence of NF.

Conditions	SPS	GUS
	nmol Frc mg^{-1} min^{-1}	*pmol MU mg^{-1} min^{-1}*
Light	7.3 (±0.7)	363 (±47)
Dark	4.4 (±0.5)	33 (±4.3)
+NF	3.4 (±0.3)	38 (±3.4)

(Fig. 3M), whereas GUS activity could be readily observed in almost the complete leaf blade of light-grown plants (Fig. 3L). When GUS activity was quantified by fluorometric analysis, it was shown that the level of *sps-gus1* expression was more than 10-fold higher in light-grown plants as compared with those grown under darkness (Table I). In 10-d-old dark-grown plants GUS activity was detected at the tip of primary leaves (Fig. 3R).

sps1 Expression in Leaves Is Regulated by the Developmental Stage of Plastids

Previous studies have reported that the expression of nuclear genes encoding photosynthesis related proteins is dependent on the developmental stage of plastids (Simpson et al., 1986; Taylor, 1989; Bolle et al., 1994; Argüello-Astorga and Herrera-Estrella, 1995; Kusnetsov et al., 1996; Kropat et al., 1997). To ascertain whether *sps1* expression is influenced by plastid development, SPS and GUS activities were determined in *sps-gus1* plants germinated in media containing norfluorazon (NF). NF is a herbicide that blocks carotenoid biosynthesis and arrests plastid development in light-grown plants (Reiss et al., 1983; Mayfield and Taylor, 1984; Simpson et al., 1986). It was found that SPS activity in the leaves of NF-treated plants was 2-fold lower than in untreated plants (Table I).

When the effect of NF on *sps-gus1* expression was analyzed, the difference between treated and untreated plants was even more pronounced. Histochemical analysis revealed no detectable GUS activity in leaves of NF-treated plants (Fig. 3N), whereas in the absence of NF a high activity was observed (Fig. 3L). When GUS activity was quantified by fluorometric assays, a 9-fold difference between NF-treated and control plants was detected (Table I).

sps1 Expression Pattern during Seed Germination

During seed germination, energy stored in the form of starch or lipids is converted into Suc, which is then transported to the germinating embryo. For the cleavage of starch stored in the endosperm of rice seeds, the biosynthesis of α-amylase and other en-

zymes (mainly hydrolytic) is induced during germination in the scutellum and aleurone of this cereal.

Because starch cleavage and Suc synthesis are required to sustain the growth of the emerging seedling, it is possible that the expression of α-amylase and SPS is coordinated. This notion is supported by the finding that the three DNA motifs responsible for the up-regulation of α-amylase genes during seed germination are also present in the 5′ flanking region of the *sps1* gene. To examine this possibility, SPS, α-amylase, and GUS activity were measured in germinating *sps-gus1* seeds during 6 d after sowing (eliminating leaves and roots when present). The level of the three enzymes showed a similar kinetic of induction with highest expression at d 6 (Fig. 6).

To determine whether light or NF influence *sps1* expression in the scutellum of germinating seeds, the GUS activity present in *sps-gus1* seeds germinated in NF containing media or grown in darkness was compared with that present in control seeds. Histochemical GUS assays of 7-d-old seedlings showed no significant differences in the expression pattern or in the relative intensity of GUS staining between these conditions (Fig. 3, O–Q). These results are in contrast with the effect of light and NF on the expression observed in leaves and indicate that in seeds *sps1* expression is independent of light and plastid development.

sps-gus1 Is Expressed in Pollen

Expression of the *sps1* promoter in other tissues of *sps-gus1* transgenic rice plants was examined by histochemical assays. Whereas no detectable activity was found in roots (Fig. 3S), GUS staining was readily detected in pollen of immature inflorescences (Fig. 3, T and U). This result suggests a possible role of SPS during pollen development.

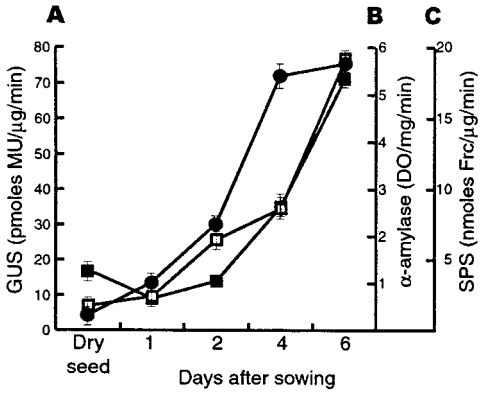

Figure 6. Kinetic of SPS, α-amylase, and *sps-gus1* expression during seed germination. GUS (A, ■), α-amylase (B, □), and SPS (C, ●) activity was determined in dry seeds and seeds of germinating transgenic s*ps-gus1* rice plants during d 6 after sowing. Most of the endosperm was removed by hand with scalpel, and activity was determined in the rest of the seed.

DISCUSSION

Tissue-Specific Expression of *sps1* Gene

SPS activity and mRNA distribution have previously been studied in the leaves and scutellum of rice (Nomura et al., 1969; Nomura and Akazawa, 1974; Valdez-Alarcón et al., 1996; Salerno et al., 1998). *sps1* mRNA was reported to be present in rice leaves by RT-PCR (Sakamoto et al., 1995; Valdez-Alarcón et al., 1996), but no mRNA could be detected in other tissues, including rice scutellum (Sakamoto et al., 1995). Here we report that the rice *sps1* is expressed not only in photosynthetic tissues, but also in pollen and in the scutellum of germinating seeds. SPS activity in the scutellum is considered to play a crucial role during seed germination by providing Suc produced from storage compounds for the developing embryo (Nomura and Akazawa, 1974). To the best of our knowledge, expression of SPS in pollen has not been reported before, and its role in this tissue is unknown. However, SPS could play a role in creating a flux gradient of carbon partitioning to pollen grains where starch formation has proven to be of importance during microsporogenesis (Lalonde et al., 1997; Mousavi et al., 1999, and refs. therein) in a similar manner as has been observed for carbon partitioning during the development of sink organs (Geigenberger and Stitt, 1991; Weber et al., 1996; Weber et al., 1997).

sps1 Expression in Leaves during Development

Levels of SPS protein in developing corn leaves has been previously reported to appear in accordance to the accumulation of phospho*enol*pyruvate carboxylase and the leaf transition from sink to source tissue (Bruneau et al., 1991). We observed that in young emerging rice leaves, *sps-gus1* is initially expressed only at the tip of the leaf, and in older leaves expression gradually extend toward the leaf base, regarding a sink-to-source transition (Turgeon, 1989; Roberts et al., 1997). These results suggest that the factors regulating sink-to-source leaf transition also regulate the expression of *sps1*.

It is interesting that in addition to the basipetal patter of expression observed in young emerging leaves, in sheathed leaves *sps1* shows a transversal gradient of expression, being higher in the outermost and lower in the innermost zone of the leaf. This pattern of *sps1* expression coincides with chlorophyll and starch localization. These results suggest that in rice leaves the sink-to-source transition occurs in two directions or that light has a major influence in the pattern of *sps1* expression during leaf development.

Previous studies have shown that in rice, as in other cereals, 60% to 90% of the total carbon accumulated in panicles is derived from CO_2 assimilated during the ripening period and that the flag leaf is the organ that contributes the most to grain filling

(Yoshida, 1981). In other reports, the level of SPS has been found to increase in the flag leaf 2 weeks after anthesis (Wada et al., 1993), indicating a relationship between seed development in the panicle and Suc synthesis in the flag leaf. Our results showed that *sps1* expression and SPS activity are not only higher in mature than in immature leaves, as would be expected according to their sink or source status, but also that the highest levels of *sps1* expression and SPS activity are present in the youngest expanded leaf. These results suggest that carbon partitioning in the youngest mature leaf of rice plants is strongly biased to Suc biosynthesis, making this leaf the major source supporting the development of immature leaves. This could be analogous to the role of the flag leaf, regarded as the major source organ for seed formation (Yoshida, 1981).

Light and Plastid Development Regulate *sps1* Tissue-Specific Expression

sps-gus1 expression was not observed in leaves of etiolated rice seedlings or in plants grown under light conditions in the presence of NF. These results show that the expression of the rice *sps1* gene in photosynthetic tissues, is regulated by light and plastid development in a similar fashion to that observed for photosynthesis-associated genes such as *rbcS*, *cab*, and *PetH* genes (Simpson et al., 1986; Green et al., 1987; Giuliano et al., 1988; Gidoni et al., 1989; Argüello-Astorga and Herrera-Estrella, 1996; Kusnetsov et al., 1996). These observations also suggest that at least some LREs present in the *sps1* promoter are functional cis-regulatory elements, and that the expression of *sps1* is to some extent coordinated with the expression of genes encoding photosynthesis-related proteins. However, the observed basipetal *sps1* pattern of expression during leaf development indicates that although light plays an important role in the regulation of this gene, its pattern of expression during leaf development is also regulated by factors determining the sink-to-source transition. Moreover, the fact that *sps-gus1* expression in seeds is independent of light or chloroplast conditions suggests that *sps1* photoresponsive regulation is specific to photosynthetic tissues and that *sps1* expression in seeds is controlled by different and independent mechanisms of regulation. In addition, GUS activity was observed in primary leaves of 10-d-old, dark-grown plants, suggesting that endogenous signals different from light can activate *sps1* expression in leaves.

We consistently observed that the difference between the SPS activity of control plants with respect to plants grown under darkness or treated with NF (Table I) was much less pronounced than that observed for the GUS activity directed by *sps-gus1*. These difference could be due to the lack of a region of *sps1* in the *sps-gus1* construct that either acts positively increasing the expression of these gene in plants grown in darkness or NF containing media or negatively in light-grown plants. Alternatively it is possible that SPS or its mRNA or both are subjected to post-transcriptional mechanisms of regulation that maintain SPS within certain limits even when *sps1* is not being transcribed.

sps1 Expression in Seeds during Germination

The concerted action of hydrolytic enzymes (i.e. α-amylase) involved in starch breakdown in the endosperm and enzymes responsible for Suc biosynthesis (i.e. SPS) in scutellum is needed to produce a transportable carbon supply to support the growth of the emerging embryo (Nomura et al., 1969). We observed that during seed germination the expression of *sps-gus1* and the accumulation of SPS follow a similar kinetic to that observed for the accumulation of α-amylase, suggesting that the expression of genes encoding enzymes involved in starch degradation and Suc biosynthesis is coordinated. The finding that the *sps1* promoter contains gibberellin-responsive elements that are conserved in the promoters of α-amylase genes (Huang et al., 1990) suggests that the expression of the *sps1* gene might be regulated by gibberellins during seed germination. It has been previously reported that SPS activity increases as a response to gibberellin treatment in soybean and spinach leaves (Cheikh and Brenner, 1992; Cheikh et al., 1992), which supports the notion of a possible regulation of *sps1* by gibberellins. Experiments to determine whether the expression of *sps1* is regulated by hormones in a similar way to that previously reported for α-amylase genes are currently being carried out in our laboratory.

MATERIALS AND METHODS

Sequencing and Identification of DNA Motifs

Sequencing was performed as in Sanger et al. (1977). Sequence homologies were determined with the FASTA program and local alignments with LALIGN, both available from Genome (Eerie, France). Manual adjustments were made to finely locate some motifs.

sps1 Gene Fusion and Plant Transformation

The *Hin*dIII-*Xho*I fragment (−1,854 to +676), which contains a 1.8-kb fragment of the promoter, followed by the untranslated leader region and two exons of the *sps1* gene, was used to construct a translational fusion with GUS by cloning in compatible sites of pBI101 (Jefferson et al., 1987), as shown in Figure 1B. The chimeric gene was subcloned in pBluescript II SK$^-$ for particle bombardment experiments (Stratagene, La Jolla, CA).

To accomplish rice (*Oryza sativa*) transformation, calli were induced by incubating mature seeds of a Taipei 309 variety on NB medium (N6 macronutrients as described by

Chu et al. (1975); B5 micronutrients and vitamins as described by Gamborg et al. (1968); Fe-EDTA as described for Murashige and Skoog medium (Murashige and Skoog, 1962); 2 μg mL^{-1} 2,4-D, 30 μg mL^{-1} Suc; 500 μg mL^{-1} Pro; 500 μg mL^{-1} Gln; 300 μg mL^{-1} casein enzymatic hydrolysate; 2.5 g L^{-1} phytaGel) for 15 d, followed by subcultures of 14 and 17 d. Calli were transferred to NBO (NB medium with 0.2 M mannitol and 0.2 M sorbitol as osmotilyte) for 4 h prior to bombardment of the *sps-gus1* construct, together with plasmid pMON410 containing the *hph* gene used as a dominant selectable marker. Bombarded calli were incubated for 16 to 20 h in the dark on NBO medium before being transferred to preselection medium (NB medium plus 30 μg mL^{-1} of hygromycin) for 7 to 10 d. Resistant cell clusters were excised and incubated for 16 to 20 d in selection medium (NB medium plus 50 μg mL^{-1} hygromycin). Resistant calli were further incubated for 9 to 11 d in preregeneration medium (NB medium plus 2 μg mL^{-1} benzylaminopurine; 1 μg mL^{-1} naphthylacetic acid [NAA]; 5 μg mL^{-1} abscisic acid; and 50 μg mL^{-1} hygromycin). Regeneration was achieved by incubation for 12 to 14 d in regeneration medium (NB medium plus 3 μg mL^{-1} benzylaminopurine, 0.5 μg mL^{-1} NAA, and 50 μg mL^{-1} hygromycin). Plants were transferred to growth medium (0.5× Murashige and Skoog salts, 0.5× B5 vitamins, 10 g L^{-1} Suc, 0.05 μg mL^{-1} NAA, and 2.5 g L^{-1} phytagel) and finally, plantlets were transferred to soil in the greenhouse. Eighty-three independent transgenic rice lines bearing the *sps-gus1* fusion were obtained. After screening for GUS activity at the tillering stage, five independent lines expressing different levels of GUS activity were selected for further analysis. The presence of the corresponding construct in the selected transgenic rice lines was confirmed by Southern-blot hybridization analysis (data not shown). Since the pattern of expression of all of the five analyzed lines was quite similar, results obtained for only one of the lines (*sps-gus1-r1*) is presented.

Plant Treatments

Rice seeds were grown in 0.7% agar (w/v) Murashige and Skoog medium and incubated at 27°C either under continuous darkness or in photoperiod (16-h light/8-h darkness). In NF treatments, 10 μM of the herbicide was added to the medium, and the growing plants were maintained in photoperiod.

Plants grown in the presence or in the absence of NF were essentially identical, although bleached and thinner with NF. Plants grown in continuous darkness were etiolated.

Fluorometric and Histochemical Assays

Protein extraction and fluorogenic reactions were performed essentially as described by Jefferson et al. (1987). GUS activity is reported as pmol of 4-methylumbelliferone per minute per mg protein.

Histochemical localization of GUS activity was achieved as described by Stomp (1992). Tissue sections of complete seedlings were treated with a vacuum pulse and incubated 12 h at 37°C. Pigments were extracted from stained tissues with methanol:acetone (3:1, v/v). Further clearing was accomplished by incubation in chloral-lactophenol (Beeckman and Engler, 1994). Starch staining was done with an iodine solution (Nakamura et al., 1995) on freshly obtained sections.

In Situ RT-PCR

Prior to conducting the in situ RT-PCR reactions, specific amplification was verified by in vitro RT-PCR, using the same reagents and conditions described below for in situ RT-PCR with the exception of the addition of 150 ng of total RNA extracted from rice leaves or embryos. *rbcS* and *sps1* mRNA could be amplified even when using an annealing temperature of 62°C in a 20-cycle PCR reaction (data not shown).

For in situ RT-PCR, the protocol was as described by Ruiz-Medrano et al. (1999). Fresh tissue sections (200 μm) were obtained with a tissue sectioner (Sorvall TC-2, DuPont Instruments, Newton, CT) or by hand in the case of seeds. Samples were incubated on a GeneAmp in situ PCR System 1000 (Perkin-Elmer Applied Biosystems, Branchburg, NJ) with a 50-μL RT-PCR reaction (0.2 μM each dATP, dCTP, and dGTP; 0.01 μM dTTP; 0.2 μM Oregon Green 488-5-dUTP [Molecular Probes, Eugene, OR]; 2.5 μM Mn $[OAc]_2$, 1× EZ buffer, and 5 units of recombinant *Thermus thermophilus* DNA polymerase [Perkin-Elmer Applied Biosystems]; 2 μM each primer). Amplification conditions involved an RT cycle at 60°C (20 min) followed by 10 PCR cycles of 94°C (30 s), 60°C (30 s), and 72°C (1 min).

Excess reagents were removed by extensive washing with 1 mM EDTA and the dUTP Oregon Green 488-5-dUTP incorporated in the amplification product was observed in situ using a confocal laser scanning microscope model TCS-4D (Leica Lasertechnik, Heidelberg) using a low intensity laser (25 mW of krypton/argon laser). The following filter sets were used: (a) fluorescein isothiocyanate, 488 nm excitation and 525 nm emission and (b) chlorophyll autofluorescence, 488 nm excitation and >620 nm emission. Image analysis and display (adjustments in contrast, brightness, etc.) were performed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

SPS Extraction and Activity Measurements

SPS activity was assayed under V_{max} substrate conditions, which implicates the use of high concentrations of substrates and the activator Glc-6-P and avoiding inorganic phosphate, to detect SPS activity in correlation to the amount of protein and independently of the activation state of the enzyme (Huber et al., 1989).

Frozen samples were ground in buffer containing 100 mM HEPES(4-[2-hydroxyethyl]-1-piperazineethanesulfonicacid), pH 7.5, 20 mM $MgCl_2$, 2 mM EDTA, 2% ethylenglycol (v/v), 20% glycerol (v/v), 20 mM β-mercaptoethanol, and 0.5 mM phenyl-methyl-sulfonyl-fluoride, and cell debris was removed by centrifugation in a microfuge at 18,000*g* for 15

min at 4°C. Endogenous metabolites were eliminated from the supernatant by passing through a Sephadex G-25 (Sigma, St. Louis) column. Five micrograms of protein was incubated in 50 μL of reaction mixtures consisting of 10 mM Fru-6-P, 10 mM UDP-Glc, 50 mM Glc-6-P, 20 mM $MgCl_2$, 100 mM HEPES (pH 7.5), and 100 mM arbutin, for 30 min at 28°C (Salerno et al., 1979, 1991). Reactions were stopped by the addition of 200 μL of 5 N NaOH. Unreacted reducing sugars were destroyed by heating 10 min at 95°C. Six hundred microliters of 2-thiobarbituric acid (Sigma) reagent was added, and the chromogenic reaction was performed as described in Percheron (1962). A control reaction where UDP-Glc was added after stopping the reaction with NaOH was included for each sample. A calibration curve using Fru as standard was performed. SPS activity is reported as nmol of Fru per minute per microgram of protein or per gram fresh weight. Protein was measured using the Bradford reagent (Bio-Rad Laboratories, San Diego).

α-Amylase Extraction and Activity Measurements

Frozen samples were pulverized using a stirrer type R2R1, (Caframo, Wiarton, ON). A 50% aliquot was used to detect GUS activity by the fluorometric assay method already described, and the remaining 50% was resuspended in 1 volume of extraction buffer [50 mM Na(Ac), pH 5.2; 3 mM $CaCl_2$; 10% glycerol (v/v)], incubated in an ice-bath during 15 min, and centrifuged at 18,000*g* during 15 min at 4°C. The aqueous phase was recovered and total protein concentration was determined using the Bradford reagent. Enzymatic activity was detected as described by Hopkins and Bird (1954), using succinic acid buffer, pH 4.5.

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