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Changes in the cellular localization of cytosolic glutamine synthetase protein in vascular bundles of rice leaves at various stages of development

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Abstract. Cellular localization of cytosolic glutamine synthetase (GS1; EC 6.3.1.2) in vascular bundles of leaf blades of rice (*Oryza sativa* L.), at the stage at which leaf blades 6 (the lowest position) to 10 were fully expanded, was investigated immunocytologically with an affinity-purified anti-GS1 immunoglobulin G. Strong signals for GS1 protein were detected in companion cells of large vascular bundles when blades 6–8 were tested. Signals for GS1 were also observed in vascular-parenchyma cells of both large and small vascular bundles. The results further support our hypothesis that GS1 is important for the export of leaf nitrogen from senescing leaves. The signals in companion cells were less striking in the younger green leaves and were hardly detected in the non-green portion of the 11th blade. In the non-green blades, strong signals for GS1 protein were detected in sclerenchyma and xylem-parenchyma cells. When total GS extracts prepared from the 6th,10th, and the non-green 11th blades were subjected to anion-exchange chromatography, the activity of GS1 was clearly separated from that of chloroplastic GS, indicating that GS1 proteins detected in the vascular tissues were able to synthesize glutamine. The function of GS1 detected in the developing leaves is discussed.

Key words: Enzyme localization – Glutamine synthetase – Nitrogen remobilization – *Oryza* – Vascular bundle

Introduction

The major source of nitrogen for developing leaves and ears in mature rice (*Oryza sativa* L.) plants is the nitrogen transported from older, senescing leaves (Mae et al. 1981). Glutamate is a major free amino acid in the leaf blades (Kamachi et al. 1991), whereas glutamine and asparagine, which is synthesized from glutamine (Lea et al. 1990), are major forms of the total amino acids in phloem sap of rice plants (Hayashi and Chino 1990). Therefore, conversion of glutamate to glutamine is required during the process for remobilization of leaf nitrogen. Glutamine synthetase (GS) is a candidate for this conversion in senescing leaves. There are two isoforms of GS in green leaves of many plants including rice leaves (Kamachi et al. 1991; Lea et al. 1990): a minor isoform located in the cytosol (GS1) and a main isoform in the chloroplast stroma (GS2). Studies with mutants lacking GS2 (Blackwell et al. 1987; Wallsgrove et al. 1987) clearly show that a major role of GS2 is the reassimilation of NH_4^+ released from photorespiration. Because the mutants were able to grow normally under nonphotorespiratory conditions (Blackwell et al. 1987; Wallsgrove et al. 1987), GS1 in leaves could be important in the synthesis of glutamine for normal growth and development. Our previous results using tissue-print immunoblots showed that the GS1 polypeptide was located in both large and small vascular bundles in all regions of the fully expanded leaf blades (Kamachi et al. 1992). On the other hand, GS2 was mainly detected in mesophyll cells. Thus, GS1 in senescing leaves is apparently responsible for the remobilization of leaf nitrogen. However, cellular localization of the GS1 within the vascular bundles of rice leaves was not established in our previous study, because of the limit of detection with the tissue-printing methods. If GS1 were truly responsible for export of leaf nitrogen, it would be expected to be localized in close proximity to the phloem in vascular tissues of senescing leaves as a functionally active form. In addition, GS1 polypeptide was detected not only in the senescing blades but also in the younger blades of rice plants (Yamaya et al. 1992). However, possible functions of GS1 in the young leaves were not described.

In this investigation, we examine the cellular localization of the GS1 protein in vascular bundles of rice-leaf blades at various stages of development by using immunocytological methods with an affinity-purified anti-GS1 immunoglobulin G (IgG), which reacts

Abbreviations: Fd-GOGAT = ferredoxin-dependent glutamate synthase; GS1 = cytosolic glutamine synthetase; GS2 = plastidic glutamine synthetase; IgG = immunoglobulin G

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monospecifically with GS1 protein in rice plants (Kamachi et al. 1992). Activity of GS1 was also estimated after its separation from GS2 on an anion-exchange column.

Materials and methods

Plant material. Rice (*Oryza sativa* L. cv. Sasanishiki) plants were grown in hydroponic culture (Kamachi et al. 1991) for 67d after imbibition in a greenhouse. Leaf blades 6 (the lowest position) to 10 (the highest position) on the main stem were fully expanded at this stage. The 11th leaf was divided into two segments, i.e. expanding green blade outside of the 10th leaf sheath and non-green leaf blade inside of the sheath. All fully expanded leaf blades and two segments of the 11th leaf blade were harvested. Fresh leaves at various positions were immediately treated for immunocytological experiments, whereas whole blades were weighed, frozen in liquid nitrogen, and stored at −80°C for biochemical experiments. At least triplicate independent samples at all leaf positions were provided for each experiment.

Immunocytology. Approximately 1-mm-thick cross-sections of the middle region of blades 6–10 were fixed with FAA solution [1.85% (v/v) formaldehyde,5% (v/v) acetic acid, and 63% (v/v) ethanol] for 48 h at 4°C. Sections of the 11th blade were also cut out from the middle part of the expanding green portion outside of the 10th leaf sheath and from that of the non-green blade inside of the sheath. The fixed tissues were sequentially dehydrated, embedded in paraffin, sliced into 10-μm sections, stretched onto a glass slide, deparaffinized, rinsed, and reacted with antibodies as described previously (Hayakawa et al. 1994). Polyclonal IgG raised against a synthetic GS1-peptide-ovalbumin conjugate (Kamachi et al. 1992), that raised against rice-leaf GS2 (Kamachi et al. 1991), and that raised against ferredoxin-dependent glutamate synthase (Fd-GOGAT; EC 1.4.7.1) purified from rice leaves (Yamaya et al. 1992), all of which had been purified with corresponding antigens (Yamaya et al. 1992), were used in the present study. Antigens on the surface of the tissue sections were visualized with ABC reagent containing AvidinDH and biotinylated horseradish peroxidase (Vectastain ABC Elite kit; Vector Lab. Inc., Burlingame, Cal., USA) as described previously (Hayakawa et al. 1994).

Separation of GS1 and GS2 activities. The frozen blades at the 6th and 10th positions and the non-green portion of the 11th leaves (two pieces of blade for each extraction: about 0.1–0.8 g FW) were ground to a fine powder in a mortar and pestle in the presence of washed quartz sand [0.3 g·(g FW)$^{-1}$] with liquid nitrogen, and then homogenized in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 5% (w/v) insoluble polyvinylpyrrolidone [5 ml·(g FW)$^{-1}$]. The homogenate was filtered through four layers of gauze and the filtrate was centrifuged at 39000·*g* for 20 min. The supernatant fraction (crude enzyme fraction) was stored in the presence of 50% (v/v) glycerol at −20°C, until required. Loss of the GS-transferase activity was not observed during a one-month storage period. The stored crude enzyme was diluted more than twofold with 25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$ and 10 mM 2-mercaptoethanol and then loaded onto a Resource Q column (Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with the same buffer. The column was washed with the same buffer until no protein was detectable in the eluate, and then GS1 and GS2 were eluted by a linear gradient of NaCl (0–350 mM) in the same buffer, based on the previous methods (Yamaya and Oaks 1988).

Two micrograms of proteins in the two peaks of GS activity were first separated by SDS-PAGE, and immunoblotting was performed with the affinity-purified GS1-specific IgG or the affinity-purified GS2 IgG as described previously (Yamaya et al. 1992).

Assays of GS activity and soluble protein. Transferase activity of GS was assayed as described previously (Hayakawa et al. 1990). One kat of enzyme activity is defined as the amount that synthesizes 1 mol γ-glutamyl hydroxamate·s^{-1} at 30°C. Soluble protein content was quantitated by the method of Bradford (1976) with bovine serum albumin as the standard.

Results

Our anti-GS1 IgG cross-reacted monospecifically with GS1 in extracts prepared from leaf blades of rice plants (Kamachi et al. 1992). Ten-micrometer cross-sections of leaf blade 6 were prepared from paraffin-embedded leaf tissues, and GS1 protein was stained with the GS1 IgG (Fig. 1). Signals of GS1 protein were clearly detected in companion cells, metaphloem-parenchyma cells, and metaxylem parenchyma cells in large vascular bundles (Fig. 1A). Particularly heavy color staining was observed in companion cells located in close proximity to the sieve elements and xylem vessel elements. In small vascular bundles, GS1 protein was detected in companion cells and metaxylem parenchyma cells, although it is hard to identify the metaphloem parenchyma cells at this magnification (Fig. 1E). The signals for GS1 were also observed in some stomata, but were very weak, when present in mesophyll cells and epidermal cells. In contrast, GS2 and Fd-GOGAT proteins were mainly located in mesophyll cells and parenchyma sheath cells (Fig. 1B, 1D). Because our GS2 antibody recognized both GS1 and GS2 in rice plants (Kamachi et al. 1991), faint signals detected with GS2 IgG in companion cells and parenchyma cells were probably caused by the GS1 protein.

Profiles for the localization of GS1 protein in large vascular bundles of the seventh and eighth blades were basically similar to that found for the sixth blade (Fig. 2B, 2C, 2D). When younger blades, such as the 9th,10th, and developing 11th green blades, were tested, the signals for GS1 in companion cells were relatively faint and xylem- and phloem-parenchyma cells were mainly stained (Fig. 2E, 2F, 2G). In non-green 11th leaf blades, on the other hand, strong signals for GS1 protein were detected in sclerenchyma and in xylem-parenchyma cells, whereas minor signals were detected in companion cells (Fig. 2H).

Since sensitive and specific methods for the staining by GS activity in situ are not available, biochemical methods were adopted to estimate whether GS1 detected in those specific cell types of vascular tissues was a functional form. The GS1 and GS2 proteins in the extracts prepared from the 6th,10th, and non-green portion of the 11th leaf blades were separated by their activities on an anion-exchange column. As shown in Fig. 3, GS1 activity eluted at about 0.12 M NaCl and was clearly separated from GS2 activity, which eluted at about 0.20 M on the chromatogram. The GS1 and GS2 polypeptides in those two peaks were confirmed by immunoblotting after the separation of the same amounts of proteins (2 μg) by SDS-PAGE, using both GS1-IgG and GS2-IgG (Fig. 3). The relative proportion of GS1 and GS2, calculated from the transferase activity in each peak fraction, was about 19:81, 10:90, and 70:30 for the 6th,10th, and the non-green 11th blades,

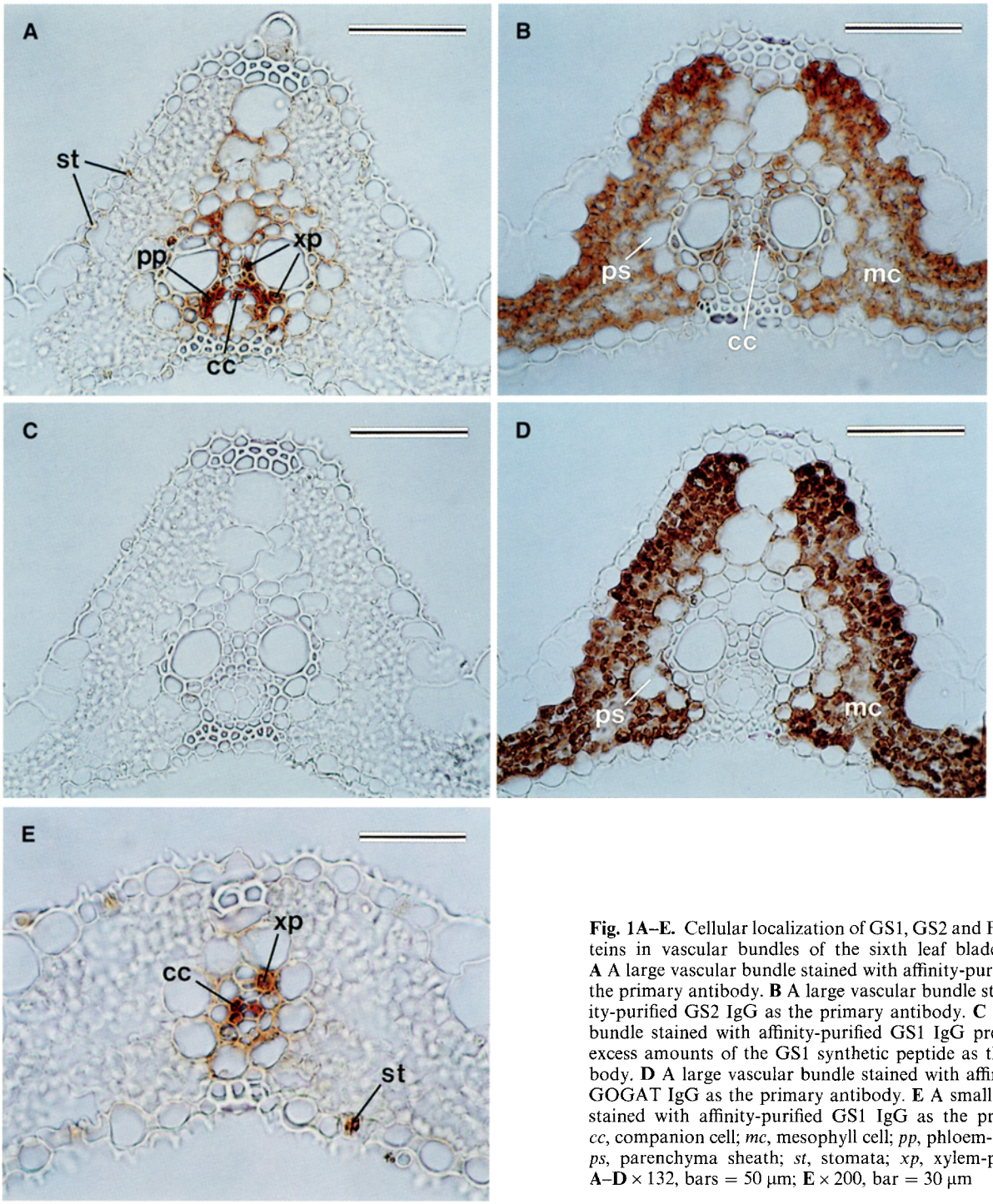

Fig. 1A–E. Cellular localization of GS1, GS2 and Fd-GOGAT proteins in vascular bundles of the sixth leaf blade of rice plants. **A** A large vascular bundle stained with affinity-purified GS1 IgG as the primary antibody. **B** A large vascular bundle stained with affinity-purified GS2 IgG as the primary antibody. **C** A large vascular bundle stained with affinity-purified GS1 IgG pretreated with an excess amounts of the GS1 synthetic peptide as the primary antibody. **D** A large vascular bundle stained with affinity-purified Fd-GOGAT IgG as the primary antibody. **E** A small vascular bundle stained with affinity-purified GS1 IgG as the primary antibody. *cc*, companion cell; *mc*, mesophyll cell; *pp*, phloem-parenchyma cell; *ps*, parenchyma sheath; *st*, stomata; *xp*, xylem-parenchyma cell. **A–D** × 132, bars = 50 μm; **E** × 200, bar = 30 μm

respectively. These results suggest that both GS1 and GS2 proteins detected immunocytologically are apparently active at the various stages of development represented by these leaf blades.

Discussion

This is the first paper describing changes in the cellular localization of GS1 in large vascular bundles of rice leaves during leaf development. The different localization in leaves of different ages suggests that the expression of GS1 protein is developmentally regulated and that, in rice, the role of GS1 in senescing leaves is probably different from that in developing leaves. The companion cells and metaphloem- and metaxylem-parenchyma cells are considered to be active in the transport of solutes, since they contain abundant mitochondria and endoplasmic reticulum (Chonan et al. 1981). Companion cells are important in the regulation of phloem loading (Van Bel 1993).

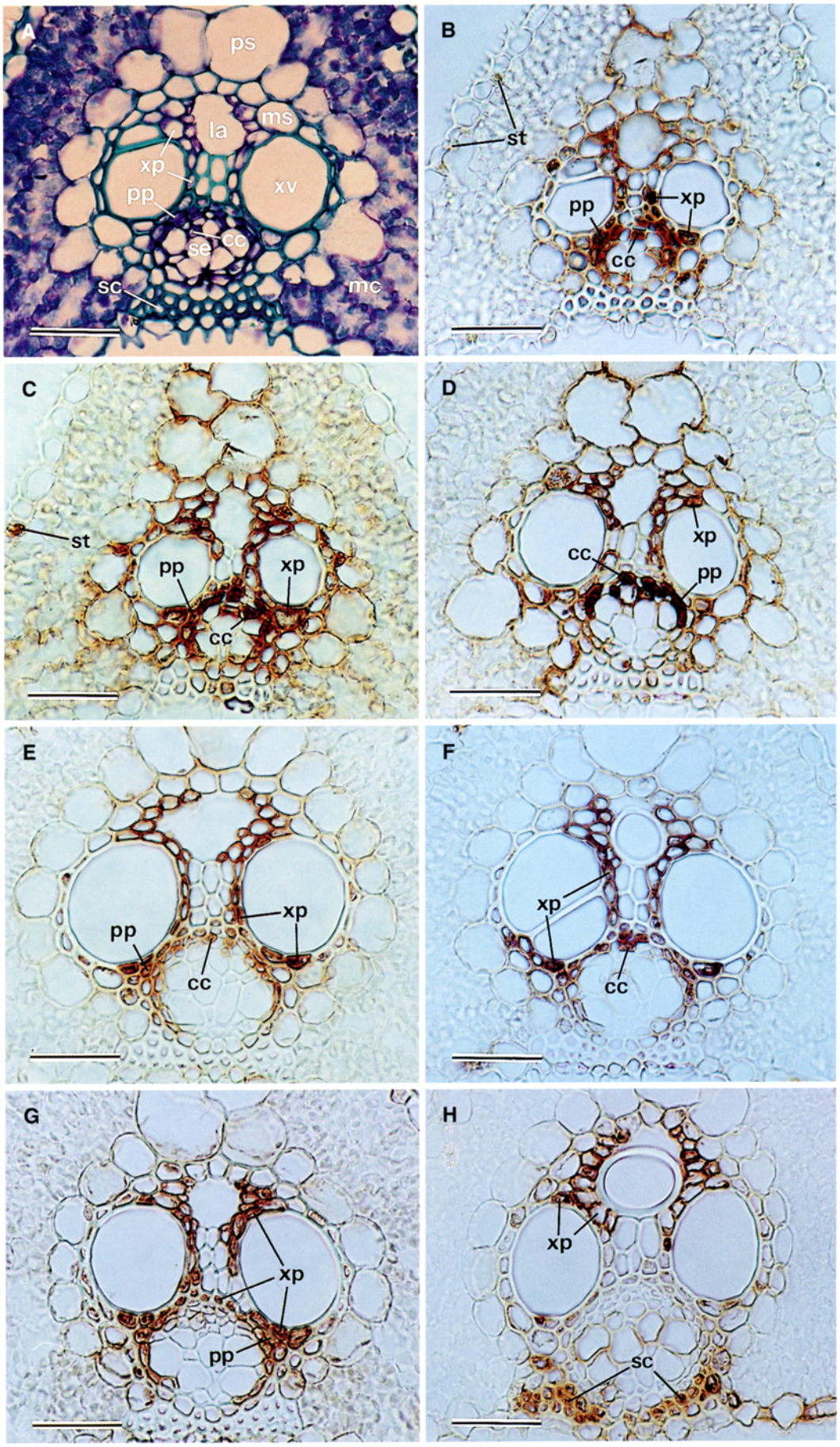

Fig. 2A–H. Cellular localization of GS1 protein in large vascular bundles of rice leaf blades at various stages of development. **A** Section of leaf 6 stained with Toluidine Blue O. **B–H** The 6th (**B**), 7th (**C**), 8th (**D**), 9th (**E**), and 10th (**F**) leaf blades, and green (**G**) and non-green (**H**) portions of the 11th blade were stained with affinity-purified GS1 IgG as the primary antibody. *la*, protoxylem lacuna; *ms*, mestome-sheath cell; *se*, sieve element; *sc*, sclerenchyma; *xv*, xylem vessel element. Other abbreviations are the same as in Fig. 1. × 132, bar = 30 μm

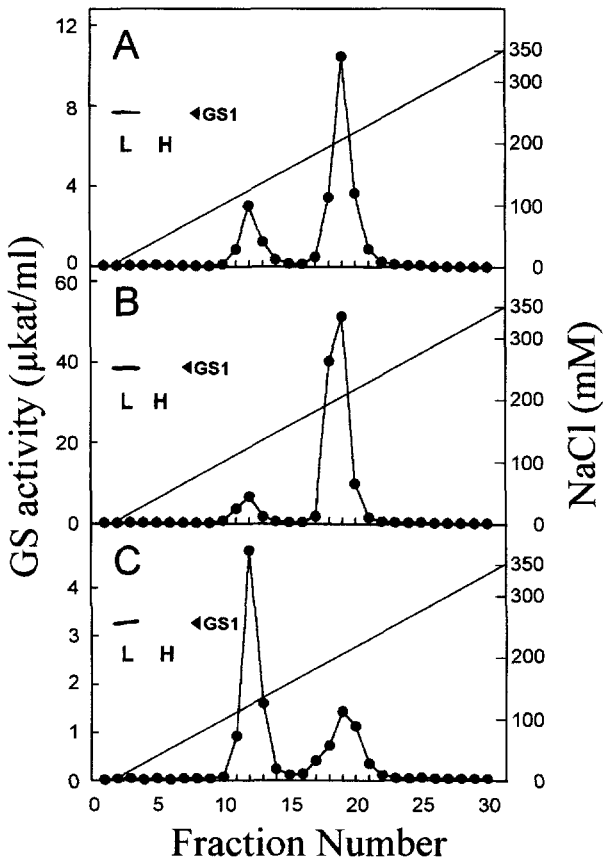

Fig. 3A–C. Elution profiles of GS-transferase activity of extracts prepared from the 6th (**A**), 10th (**B**), and non-green portion of the 11th (**C**) blades of rice leaves. Total activities loaded on the anion-exchange column were 29, 136, and 17 nkat for the 6th, 10th, and 11th blades, respectively. In each case, more than 77% of the total activity was recovered after elution. Triplicate analyses with independent samples were performed for separation of GS1 and GS2, and typical profiles are indicated. Immunoblots of GS1 with affinity-purified GS1-IgG were carried out after the separation of 2 μg of proteins into two peaks, eluted with low (**L**) and high (**H**) salt concentrations, by SDS-PAGE

Localization of GS1, as an active enzyme, in companion cells of vascular bundles of relatively old leaf blades further supports our hypothesis (Kamachi et al. 1992) that GS1 is important in the synthesis of glutamine, which is a major form of nitrogen exported from the senescing rice leaves (Hayashi and Chino 1990). It is known that the parenchyma cells of metaxylem, metaphloem, and companion cells are interconnected by plasmodesmata and that the solutes, which are transported via phloem, finally accumulate in companion cells (Chonan et al. 1981). Companion-cell-specific localization of GS1 was also shown using immuno-gold labeling by a Portuguese group working with *Nicotiana tabacum* (Carvalho et al. 1992) and *Solanum tuberosum* (Pereira et al. 1992), although they referred to neither its localization in vascular parenchyma cells, its activity, nor changes in the labeling profile according to the leaf age. Another approach to determine the localization of cytosolic GS has been to perform molecular-genetic analyses. At present, however, it seems difficult to propose a general model for GS gene expresssion based on experiments with transgenic plants, because the expression of GS gene in its native background is not necessarily the same as the expression of GS promoter-reporter gene constructs introduced into heterologous hosts. For example, Edwards et al. (1990) showed that the promoter for cytosolic GS of pea nodules was expressed within the phloem elements of transgenic tobacco leaves; however, the GS has not been shown to be expressed in the vascular tissues of pea. Marsolier et al. (1995) clearly pointed out the complexity of gene expression for GS21: the gene is nodule-specific in soybean, its native background, but when the promoter-reporter gene constructs are introduced into *Lotus corniculatus* the chimeric gene is specifically expressed in the vasculature of all the organs in the transgenic plants.

In relatively young blades, such as the 9th and 10th blades, and the green portion of the 11th blade of rice plants, metaphloem- and metaxylem-parenchyma cells were the major compartment in which GS1 protein was located. When the metabolic route for generation of NH_4^+, such as photorespiration, nitrite reduction, protein catabolism, and so on (Lea et al. 1990), is considered in those metaxylem- and metaphloem-parenchyma cells, a specific function for GS1 at this leaf age is not yet easily understood. A function for the GS1 detected in stomata should also be considered in the future.

In the developing non-green blade of the 11th leaf, the signals for GS1 protein are mainly detected in sclerenchyma cells and xylem-parenchyma cells. The secondary cell wall is actively forming at this stage of leaf development (Chonan et al. 1981). Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), the key enzyme for biosynthesis of lignin polymer, generates NH_4^+, and the bean *PAL2* gene has recently been shown to be expressed in the early stages of vascular development (Leyva et al. 1992). Cytosolic GS in the young vascular tissues of rice leaves may be responsible for the assimilation of NH_4^+ derived from the PAL reaction. Recent observations by Razal et al. (1996), using ^{15}N-NMR analyses, indicated that NH_4^+ released by PAL was first incorporated into the amide nitrogen of glutamine in potato discs. Also, GS1 may function to assimilate NH_4^+ derived from the reaction of asparaginase which is known to be expressed in young developing tissues of transgenic tobacco plants (Grant and Bevan 1994). Asparagine, as well as glutamine, is also the major form of nitrogen-transport compound in phloem solutes of rice plants (Hayashi and Chino 1990).

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