Immunolocalization of a Unique Form of Maize Kernel Glutamine Synthetase Using a Monoclonal Antibody

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The pedicel (basal maternal tissue) of maize (Zea mays L.) kernels contains a physically and kinetically unique form of glutamine synthetase (GS<sub>p1</sub>) that is involved in the conversion of transport forms of nitrogen into glutamine for uptake by the developing endosperm (M.J. Muhitch [1989] Plant Physiol 91: 868–875). A monoclonal antibody has been raised against this kernel-specific GS that does not cross-react either with a second GS isozyme found in the pedicel or with the GS isozymes from the embryo, roots, or leaves. When used as a probe for tissue printing, the antibody labeled the pedicel tissue uniformly and also labeled some of the pericarp surrounding the lower endosperm. Silver-enhanced immunogold staining of whole-kernel paraffin sections revealed the presence of GS<sub>p1</sub> in both the vascular tissue that terminates in the pedicel and the pedicel parenchyma cells, which are located between the vascular tissue and the basal endosperm transfer cells. Light staining of the subaleurone was also noted. The tissue-specific localization of GS<sub>p1</sub> within the pedicel is consistent with its role in the metabolism of nitrogenous transport compounds as they are unloaded from the phloem.

In developing seeds, assimilates are unloaded from the phloem and pass symplastically through cells of maternal origin before being unloaded into the apoplastic space that separates the maternal tissue from the developing endosperm (Thorne, 1985). The maternal tissues of developing seeds play a dynamic role in supplying the seed with nitrogen and carbon assimilates. Porter et al. (1987), adapting the empty ovule method developed for legumes, observed that Gln was the predominant amino acid released from the pedicel (basal maternal tissue) of the maize (Zea mays L.) kernel. Moreover, comparison of the free amino acids of the cob vascular sap and the pedicel (Lyznik et al., 1982), as well as studies utilizing radiolabeled amino acids (Muhitch, 1993, 1994), suggest that the pedicel is the primary site for maize kernel Gln synthesis.

In maize, as well as in other higher plants, GS is represented by several isozymes that are differentially expressed in various tissues and organs (Lara et al., 1984; Mack and Tischner, 1990; Green and Wong, 1992; Muhitch and Felker, 1994). Higher plant GS isozymes are encoded by small gene families (Cullimore et al., 1984; Tingey et al., 1987; Sakamoto et al., 1989; Sakakibara et al., 1992; Li et al., 1993). These GS isozymes are active in root and leaf primary ammonia assimilation, in leaf photorespiration, and in storage organ, root nodule, and senescent tissue Gln synthesis for intercellular transport (Kawakami and Watanabe, 1988; Coruzzi, 1991). In developing maize kernels, most of the GS is found in the pedicel region (Muhitch, 1988) as a pair of isozymes, one of which (GS<sub>p2</sub>) is a physically and kinetically unique form of maize GS (Muhitch, 1989). The relatively high K<sub>m</sub> of GS<sub>p1</sub> for glutamate suggests that the role of this GS isozyme may be to catalyze net synthesis of Gln for uptake by the developing endosperm (Muhitch, 1989). An immunohistochemical examination of GS distribution within the maize kernel using a polyclonal antibody raised against purified spinach GS (Ericson, 1985) revealed strong staining in the vascular tissue of the pedicel as well as in the pericarp parenchyma surrounding the base of the endosperm (Felker and Muhitch, 1990). However, the antibody used in that study did not differentiate between the two pedicel GS isozymes. To elucidate more fully the role of GS<sub>p1</sub> in maize kernel nitrogen metabolism, we have raised a monoclonal antibody against a purified pedicel GS preparation that recognizes the GS<sub>p1</sub> isozyme but not GS<sub>p2</sub>. Here we report on the tissue-specific localization of the GS<sub>p1</sub> isozyme using this antibody.

MATERIALS AND METHODS

Purification of GS<sub>p1</sub>

Maize (Zea mays L.) inbred OH43 was grown in a greenhouse and hand pollinated. Kernels were harvested 15 to 25 d after pollination and the pedicels were removed by dissection, frozen in liquid nitrogen, and stored at −80°C. Maize pedicel GS was partially purified by anion-exchange, hydroxyapatite, and phenyl-Sepharose chromatography as described previously (Muhitch, 1989). Pooled fractions were then concentrated using a pressurized stirred cell and loaded onto a 50 × 1.6 cm column of Superose 6 gel filtration media (Pharmacia).<sup>1</sup> The column

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<sup>1</sup> Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other vendors that might also be suitable.

Abbreviations: GS, Gln synthetase; TBST, Tris-buffered saline containing 0.05% Tween 20.
Immunoprecipitation experiments were carried out as described previously (Felker and Muhitch, 1990).

Tissue Printing

Tissue prints of maize kernels harvested 20 d after pollination and bisected longitudinally were prepared as described by Cassab and Varner (1987). After allowing the nitrocellulose to dry, it was immersed in TBST and processed as described above for immunoblots.

Immunohistochemical Localization

Median sagittal sections, 1 mm thick, were cut from A636 corn kernels harvested 22 d after pollination and fixed for

Figure 1. Immunoblots of partially purified pedicel GS probed with HL699 (lane 1) and HL702 (lane 2). The lower and upper immunostaining bands correspond to the subunits of GS_{p1} and GS_{p2}, respectively (Muhitch, 1989).

Production and Screening of Monoclonal Antibodies

Monoclonal antibodies were prepared against the purified maize kernel GS_{p1} at the University of Florida Hybridoma Core Laboratory using standard methods (Galfre and Milstein, 1981). ELISA screening and western blotting was done on a total of 400 hybridoma supernatants using appropriate maize tissue extracts to allow visualization of the two maize pedicel GS polypeptides. Fifty-five supernatants were judged positive based on their strong cross-reactivity with the appropriate mol wt subunit bands from crude extracts as well as with the purified pedicel GS protein subunits. Production of ascites fluid and purification of the immunoglobulins by affinity chromatography on protein A Sepharose was restricted to two clones, HL699 and HL702.

PAGE and Immunoblotting

Denatured proteins were separated on SDS-containing 12.5% polyacrylamide gels and electrophoretically transferred to nitrocellulose overnight using a Transphor unit (Hoefer Scientific Instruments, San Francisco, CA). The nitrocellulose was then blocked in 3% nonfat dry milk in TBST and incubated for 1 h at room temperature with a 1:3000 dilution of monoclonal antibody. After washing with TBST, the nitrocellulose was incubated for 1 h at room temperature in a 1:1000 dilution of anti-mouse IgG-alkaline phosphatase conjugate (Sigma). After further washing, the blots were developed using 0.1% each of naphthol AS-BI phosphate and Fast Blue RR in 100 mM Tris (pH 9.2) and stopped with 70% ethanol.

Figure 2. Immunoprecipitation of pedicel isozymes GS_{p1} or GS_{p2} with monoclonal antibody HL699. Top, Separation of pedicel GS isozymes by anion-exchange chromatography; GS was determined by the semibiosynthetic assay (Muhitch, 1988). Bottom, Immunoprecipitation of separated pedicel isozymes by various amounts of the HL699 antibody. Portions of the supernatant were assayed using the transferase assay (Muhitch, 1989). One hundred percent activity = 5.1 and 18.1 μmol γ-glutamyl-hydroxamate formed min^{-1} mg^{-1} protein for GS_{p1} and GS_{p2}, respectively.
Monoclonal Antibody That Specifically Recognizes GS<sub>p1</sub>

Monoclonal antibodies were raised against a partially purified preparation of maize pedicel GS<sub>p1</sub> that was largely, but not completely, devoid of GS<sub>p2</sub>, a second isozyme that is also present in the pedicel (Muhitch, 1989). The hybridoma supernatants were screened against the same purified maize kernel pedicel GS preparation used to raise the antibodies, looking for recognition of the slightly lower-M<sub>r</sub> subunit, which corresponds to GS<sub>p1</sub>, without corresponding recognition of the slightly larger-M<sub>r</sub> subunit, which corresponds to GS<sub>p2</sub>. Of several hundred preparations that were screened, only one, designated HL699, was found to meet this criterion (Fig. 1). Further evidence for the specificity of HL699 for GS<sub>p1</sub> was shown by immunoprecipitation experiments. The two pedicel isozymes were separated from each other by anion-exchange chromatography of a clarified pedicel homogenate (Fig. 2, top). Incubation of aliquots from early-eluting GS<sub>p1</sub> fractions and late-eluting GS<sub>p2</sub> fractions with increasing amounts of HL699 overnight, followed by centrifugation, resulted in the complete removal of GS<sub>p1</sub> activity, whereas GS<sub>p2</sub> activity was unaffected (Fig. 2, bottom).

HL699, which recognizes only the lower-M<sub>r</sub> subunits corresponding to GS<sub>p1</sub>, and HL702, which recognizes the subunits of both forms of pedicel GS, were used to probe immunoblots containing SDS-PAGE-separated proteins from various tissues of maize as well as from maize endosperm-derived suspension cultures. HL702 cross-reacted with protein bands in the root, leaf, endosperm, and suspension cultures and reacted strongly with the protein subunits in the pedicel lane (Fig. 3). Interestingly, HL702

Figure 4. Tissue prints of whole maize kernel probed with HL702 (A) or HL699 (B). Impressions for A and B were made with opposite halves of the same kernel. The bar represents 1 mm.
Figure 5. Immunohistochemical localization of GS in maize kernel tissues. A, D, and G, Main pedicel vascular bundle. B, E, and H, Pedicel parenchyma. sp, Outer spongy parenchyma; pp, pedicel parenchyma; e, basal endosperm. C, F, and I, Pericarp. p, Pericarp; e, endosperm. A, B, and C, Control. D, E, and F, Probed with HL699. G, H, and I, Probed with HL702. The bar represents 0.1 mm. For photographs of the controls, the outline of the cell walls was accentuated by reducing the condenser aperture.
did not recognize the higher-\( M \) subunit associated with the chloroplast form of the enzyme. In contrast to HL702, the HL699 monoclonal antibody did not react with the leaf, root, or suspension culture lanes but reacted strongly with the pedicel sample and very weakly with the endosperm sample.

### Tissue Prints of Maize Kernels

When tissue prints of developing maize kernels were probed with the nonspecific anti-GS monoclonal antibody HL702, all kernel tissues, including the endosperm, the embryone, and the pedicel tissues, were stained (Fig. 4). Staining was particularly pronounced within the pedicel region, which is consistent with the earlier biochemical observation that the pedicel contains relatively high levels of GS activity compared to the other kernel tissues (Muhitch, 1988). In contrast, HL699 labeled only the pedicel and the pericarp surrounding the lower endosperm. Within the pedicel, staining was uniform, suggesting that GS\(_{p1}\) is evenly distributed throughout the region. Prints with either no primary antibody or an irrelevant primary antibody were devoid of staining (not shown).

### Silver-Enhanced Immunogold Staining of Maize Kernel Sections

Figures 5, A to C, and 6, A to C, represent control treatments of vascular bundle, pedicel, pericarp, endosperm, aleurone, and embryo tissues, all of which were devoid of silver staining. With the monoclonal antibody HL699, a strong positive signal was observed in the main vascular strand of the pedicel (Fig. 5D). Staining of the vascular strand was somewhat more intense and slightly broader with monoclonal antibody HL702 (Fig. 5G). Intense staining of the pedicel parenchyma cells was also obtained with both antibodies, although the signal was much more discretely confined to the pedicel parenchyma with HL699, whereas HL702 strongly stained cells in the underlying spongy parenchyma as well (Fig. 5, E and H). Both antibodies also stained the adjacent basal endosperm transfer cells (Fig. 5, E and H). In contrast to the strong staining of the basal kernel tissues, both monoclonal antibodies stained only scattered single cells within the pericarp (Fig. 5, F and I).

Within the central endosperm, HL702 exhibited strong staining, whereas HL699-dependent staining was not above control levels (Fig. 6, D and G). Surprisingly, subaleurone cells within the lower endosperm were strongly stained by HL699 as well as by HL702 (Fig. 6, E and H). The embryo was intensely stained by HL702, but was devoid of HL699-dependent staining (Fig. 6, F and I).

The contrast in kernel staining patterns between HL699 and HL702 shows conclusively that GS\(_{p1}\) is expressed in specific cell types within the developing maize kernel. Staining of the sub-aleurone and basal endosperm transfer cells by the GS\(_{p1}\)-specific monoclonal antibody HL699 explains the low levels of cross-reactivity of the antibody with whole endosperm samples on immunoblots (Fig. 3) and definitively shows that although the majority of GS\(_{p1}\) is found within the pedicel, it is not confined strictly to this tissue. Association of cytosolic GS isoforms with vascular tissues has been previously demonstrated for rice (Kamachi et al., 1992), potato (Pereira et al., 1992), and tobacco (Carvalho et al., 1992) leaves. Moreover, the promoter region of a cytosolic GS gene from pea directs the expression of a reporter gene specifically within the vascular elements in transgenic tobacco (Edwards et al., 1990). Because the presumed role of vascular-associated GS is synthesis of Gln for intercellular transport (McGrath and Coruzzi, 1991), the presence of GS\(_{p1}\) at the phloem terminal of the vascular bundles leading to the maize kernel is curious, since nitrogen compounds are being unloaded rather than loaded at this location. Because the maize pedicel has been shown to contain relatively high amounts of both glutamate (Lyznik et al., 1982) and ammonia (Raczynska-Bojanowska et al., 1986), GS\(_{p1}\) could serve to enhance pedicel Gln levels for efficient active transport of nitrogen into the endosperm (Lyznik et al., 1989; Ugalde and Jenner, 1990; Muhitch, 1993). Within the aleurone cells, GS\(_{p1}\) may also catalyze net Gln synthesis, thus helping to meet the strong demand for Gln in storage protein synthesis. Given the high \( K_m \) of GS\(_{p1}\) for glutamate (Muhitch, 1989), this isozyme could serve to catalyze the net synthesis of Gln in these tissues without depleting the glutamate necessary for operation of the glutamate synthase cycle or for transamination reactions.

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### LITERATURE CITED


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Tingey SV, Walker EL, Coruzzi GM (1987) Glutamine synthetase genes of pea encode distinct polypeptides which are differentially expressed in leaves, roots and nodules. EMBO J 6: 1–9