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Nitrogen Metabolism in the Stalk Tissue of Maize

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ABSTRACT

During ear development in maize (*Zea mays* L.), nitrogenous compounds are translocated from vegetative organs to the kernels. At anthesis, the stalk contains approximately 40% of the total plant N, and contributes 45% of the N remobilized to the ear. Therefore, the stalk has an important function as a temporary reservoir for N. Little is known of the metabolism of maize stalks, and this paper describes initial studies of enzymes of N metabolism. High *in vitro* activity of glutamine synthetase (GS) in maize stalk samples throughout ear development contrasted with a peak in activity of glutamate synthase soon after anthesis and negligible nitrate reductase. With fresh sections of stalk tissue collected at anthesis, ^{15}N-feeding experiments confirmed high GS and low nitrate reductase activities. Two isoforms of GS were separated from extracts from stalk tissue: GS1, the cytoplasmic form, increased to maximum levels at 2 weeks postanthesis and remained fairly high thereafter; whereas the plastidic form, GS2, declined progressively during kernel development. Western blot analysis confirmed the presence of constantly high levels of GS protein after anthesis. The levels of GS proteins decreased after transfer of N-starved, hydroponically grown plants to N-rich conditions in order to restrict remobilization of N. In contrast, transfer of plants grown under abundant N conditions to N-free medium, which encourages N remobilization, resulted in a relative increase in GS protein. Because glutamine is the major form of N transported in maize, the results indicate that GS, specifically the GS1 isoform, has a central role in the remobilization on nitrogenous compounds from the stalk to the ear.

Previous studies of the partitioning of N in maize showed that the stalk contains about 40% of total plant N at anthesis (2, 4, 20). After anthesis, substantial amounts of N are translocated from vegetative organs to the ear, and some work has indicated that leaves contribute 65 to 85% of the N remobilized to the grain (4, 20), whereas recent work showed that stalk and leaves both contribute 45% of total remobilized N, with a 10% contribution from roots (our unpublished data). Clearly, at least in some circumstances, the stalk is an important temporary reservoir for N.

In the process of N remobilization, proteins are hydrolyzed and amino acids are converted into form(s) that can be translocated to developing organs (11). In many higher plants, including maize, glutamine is the major form in which N is remobilized (1, 14). However, little work has been done on the N metabolism of maize stalks, especially after anthesis. In this paper, initial studies on N metabolism in maize with special reference to the stalk, to GS[2], and to events during the remobilization of N to the ear are reported.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L.) genotypes B73Ht × LH51 and Pioneer 3377 were grown either under field conditions (with 269 kg N/ha applied before planting) at Cranbury, NJ, in 1989, or under hydroponic conditions (Hoagland nutrient solution, 15 mM NO_3^-) in a growth chamber (30–25°C and 16/8 h day/night, 300 $\mu mol/m^2 \cdot s$, and 70% RH).

In experiments to follow the changes of GS protein from stalk tissue as affected by the manipulation of N remobilization, the plants grown under abundant N conditions (15 mM N solution renewed every 2 weeks) until anthesis were transferred to N-free solution to stimulate N remobilization (our unpublished data). To suppress N remobilization, plants grown under deficient N conditions (2 mM N without renewing) until anthesis, were transferred to abundant N conditions (15 mM N, our unpublished results).

Enzyme Extractions and Assays

The internode subtended by the primary ear shoot was harvested, sectioned, pulverized in liquid N_2, and stored at −80°C pending analyses. The frozen samples were homogenized in buffer (3 g fresh weight/mL) with a Polytron. For extraction of GS, GOGAT, and GDH, the buffer was 50 mM Tris-HCl at pH 7.6 containing 1 mM EDTA, 10 mM $MgCl_2$, and 3 mM DTT; for NR, the buffer contained 0.2 M Tris-HCl at pH 8.2, 1 mM EDTA, 1 μM sodium molybdate, 5 μM flavin adenine dinucleotide, 3 mM DTT, and 1.5% BSA. The homogenate was centrifuged at 20,000*g* for 15 min. The supernatant was applied to a small G-25 column (PD10, Pharmacia) that had been preequilibrated with the extraction buffer, and the enzymes were collected for activity assays.

GS was assayed by the semi-synthetic reaction with hydroxylamine as substrate by the method of Rhodes *et al.* (15). NADH-dependent GOGAT and GDH were assayed by following the oxidation of cofactor NADH according to Misra and Oaks (12) and Joy (8), respectively. NR was measured as described by Scholl *et al.* (16). Protein contents were estimated from the TCA precipitate (3).

Western Blot Analysis of GS Protein

Proteins were extracted from stalk tissues with 50 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA, 10 mM $MgCl_2$,

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[2] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; NR, nitrate reductase; MSX, methionine sulfoximine.

Table I. *In vitro NR and GS Activities in Ear Leaf and Ear Stalk of Maize (B73 × LH51) Grown under Hydroponic Conditions*

Growth Stage	NR		GS	
	Leaf	Stalk	Leaf	Stalk
d from anthesis	*μmol/mg protein·h*			
−14	0.30 ± 0.02	0.16 ± 0.01	8.7 ± 0.2	21 ± 0.4
0	0.23 ± 0.01	0.05 ± 0.01	9.3 ± 0.2	24 ± 0.7
14	0.14 ± 0.01	0.00	9.1 ± 0.4	30 ± 0.5
28	0.06 ± 0.01	0.00	7.7 ± 0.7	24 ± 1.2
42	0.00	0.00	6.5 ± 0.8	18 ± 1.9

and 3 mM DTT. Denaturing polyacrylamide electrophoresis of the proteins was performed in 12.5% gels (10). Proteins from the gels were electrotransferred to nitrocellulose membranes. Immunoblots were performed with rabbit antiserum prepared against both forms of GS purified from *Pinus banksiana* Lamb (kindly provided by Dr. L. P. Vézina, Agriculture Canada, Québec, Canada, [24]). Immunodetection (7) was performed with the immunoglobulin G raised against GS and visualized by goat anti-rabbit horseradish peroxidase development method (7). Only the major band (GS1) was scanned for quantification. The band corresponding to GS2 was hardly detected and therefore was not analyzed.

GS Isozyme Separation

Protein extraction was performed as described above. The desalted protein solution was applied to an anion exchange column (Fast Protein Liquid Chromatography Mono Q, Pharmacia), and proteins were eluted with a NaCl gradient (0–300 mM) in the extraction buffer. The GS activities of eluted fractions were assayed by the semi-synthetic method described above.

Kinetic Parameters of GS Isozymes

For studying kinetic parameters of GS isoforms from leaf and stalk tissues, the protein extract (see above) was partially purified by ammonium sulfate precipitation (30–60% saturation fraction retained), followed by desalting on a G-25 column, absorption on a hydroxylapatite column, and separation on an anion exchange column (Fast Protein Liquid Chromatography Mono Q, Pharmacia) as described above.

To determine the pH optimum of GS, Tris-HCl or -base buffer (pH 6–8.5) was used in a reaction medium that contained all the substrates at near saturation. The apparent K_m values for glutamate, ATP, and NH_2OH were determined by assaying GS activity at various substrate concentrations. One substrate concentration was varied, whereas the others were kept at near saturation. Lineweaver-Burk double reciprocal plots were used to calculate the apparent K_m values.

^{15}N Feeding Analysis

Fresh stalk sections (1 mm thick) from the ear internode of a greenhouse-grown plant of B73Ht × LH51 were vacuum-infiltrated with 1 mM MSX, a specific inhibitor of GS (22), or water (control) for 15 min, and ^{15}N-labeled NO_3^- as KNO_3,

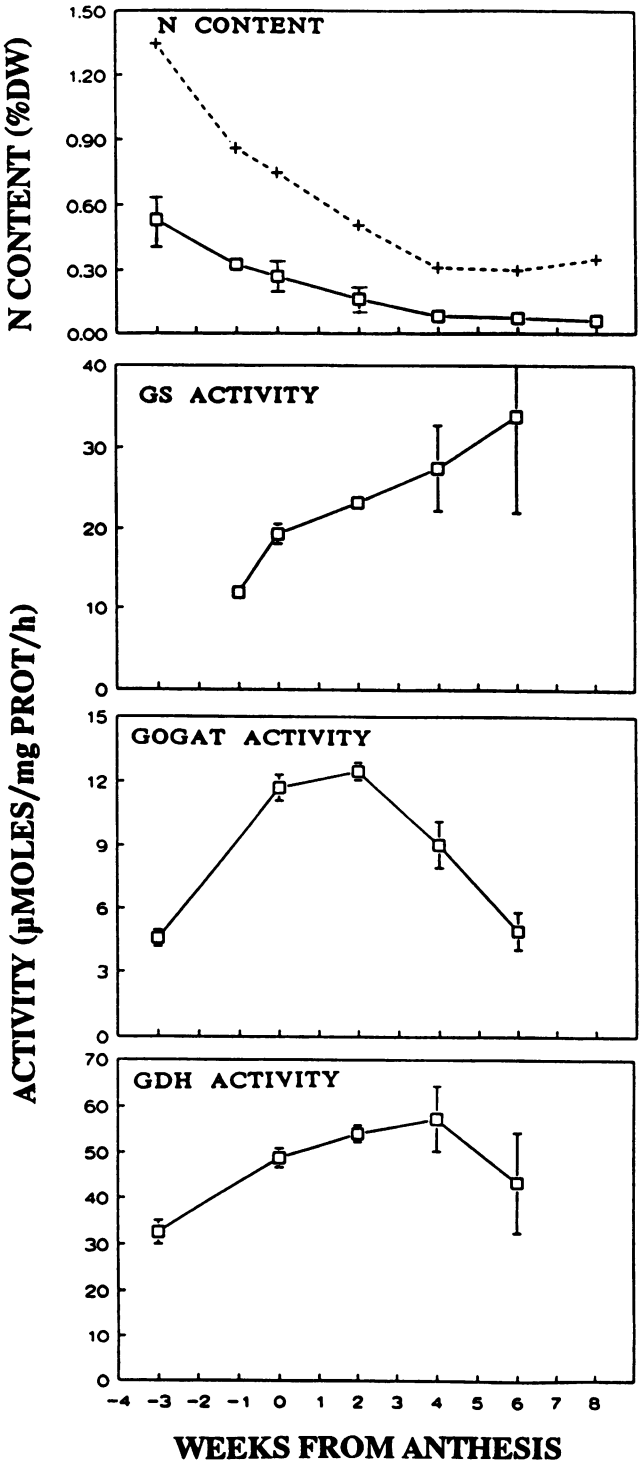

Figure 1. Developmental changes in N, GS, GOGAT, and GDH activities of ear internode stalk of maize plants (B73 × LH51) grown under field conditions with 269 kg N/ha from 3 weeks preanthesis to 6 weeks postanthesis. + indicates total N content and □ indicates total soluble protein-N content. Bars indicate the standard errors of triplicate samples. Where error bars are not shown, the error was smaller than the symbol used for graphing.

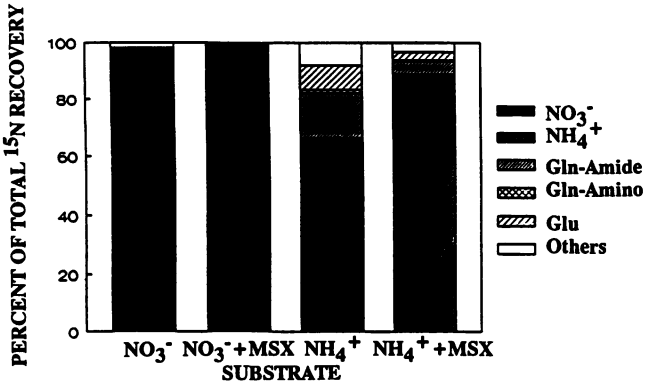

Figure 2. Incorporation of ^{15}N from labeled nitrate or ammonium into different nitrogenous compounds in stalk slices of maize (Pioneer 3377) grown under hydroponic conditions with Hoagland nutrient solution containing 15 mM NO_3^-. One-millimeter slices of the ear internode were vacuum infiltrated with 1 mM of MSX or H_2O (control) at room temperature for 15 min; labeled ^{15}N-nitrate or ammonium was added to a final concentration of 5 mM. After 1 h incubation, the tissues were collected, washed several times with H_2O, and extracted for ^{15}N analysis. Gln-Amide and Gln-Amino denote the amide and amino group of glutamine, respectively.

and NH_4^+ as $(NH_4)_2SO_4$ (99% atom excess) were added to a final concentration of 5 mM N and incubated at room temperature for 1 h. The slices were then collected, washed several times with deinonized water, extracted by homogenizing in 70% ethanol, and analyzed for distribution of ^{15}N among amide-labeled glutamine, amino-labeled glutamine, glutamate, aspartate, and other amino acids (21).

RESULTS AND DISCUSSION

Enzyme Activities

In vitro assays of NR and GS activities from both ear leaf and ear stalk of B73Ht × LH51 grown under hydroponic conditions showed a trend of decreasing NR activity with time (Table I). Whereas stalk NR activity was detected only prior to and at anthesis, leaf NR activity was detectable until 4 weeks postanthesis. In contrast, GS activity peaked at or shortly after anthesis and a significant amount was still present by 6 weeks postanthesis in both leaf and stalk (Table I).

With plants (B73Ht × LH51) grown under field conditions, the *in vitro* GS specific activity in their stalks increased threefold during the period of 1 week before anthesis until 6

Table II. *Functional Properties of GS Isoforms in Stalk and Leaf of Maize (B73 × LH51)*

Property	Stalk		Leaf	
	GS1	GS2	GS1	GS2
pH optimum	7.4	7.4	7.4	7.4
K_m (mM)				
Glu	6.5	10	3.5	5.0
NH_2OH	0.75	2	0.2	0.2
ATP	2.0	5	1.2	1.5

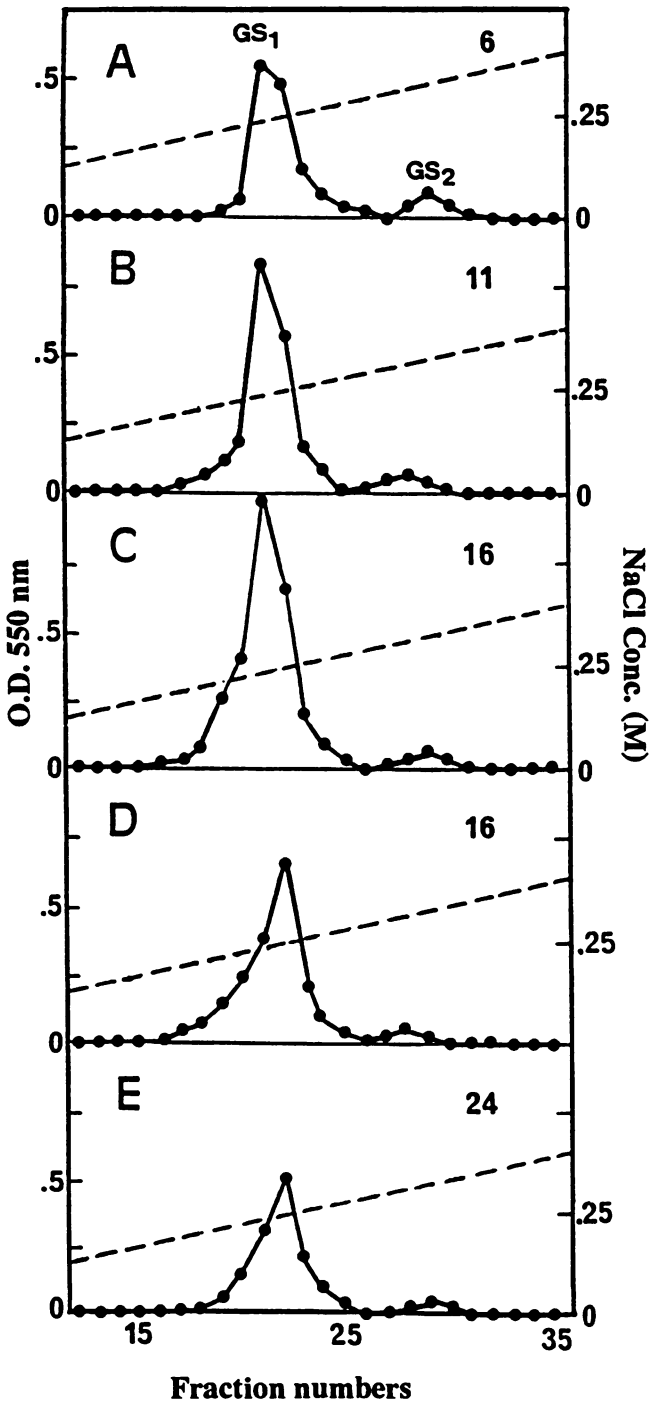

Figure 3. Developmental patterns of GS isoforms in the ear internode stalk of maize (Pioneer 3377) grown under field conditions with 269 kg N/ha. A, 3 weeks preanthesis; B, anthesis; C, 2 weeks postanthesis; D, 4 weeks postanthesis, and E, 6 weeks postanthesis. Values in top right of each panel represent the ratios of GS1 to GS2 activities. Amounts of proteins derived from similar fresh weights of tissue were applied to the anion exchange column.

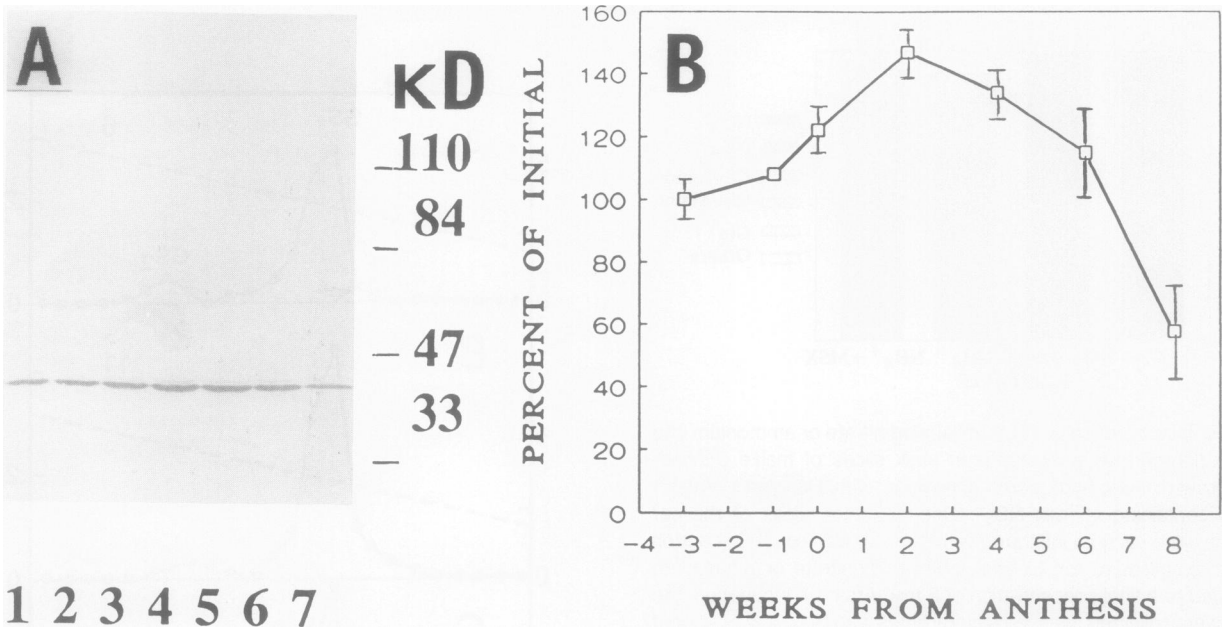

Figure 4. A, Western blot analysis of GS protein from ear internode stalk of maize (B73 × LH51). Equal amounts of proteins from stalk extracts were loaded in each well, electrophoresed on 12.5% SDS-PAGE, transferred to nitrocellulose membrane, and probed with rabbit anti-serum against pine GS. Lane 1, 3 weeks preanthesis; lane 2, 1 week preanthesis; lane 3, anthesis; lane 4, 2 weeks postanthesis; lane 5, 4 weeks postanthesis; lane 6, 6 weeks postanthesis; lane 7, 8 weeks postanthesis. Lane kD shows molecular mass standards. B, Gel scan analysis of blot as percentage of initial value at 3 weeks preanthesis.

weeks after anthesis (Fig. 1). During this period of time, nitrogenous compounds were progressively remobilized from the stalk (Fig. 1). These GS activities were about two- to threefold higher than those in the leaf at respective stages (data not shown).

Stalk GOGAT activities peaked at about 2 weeks postanthesis and declined thereafter (Fig. 1). In the same tissue, GDH activities peaked at 4 weeks postanthesis and declined only slightly thereafter (Fig. 1). NR activity in the field-grown stalk tissue was very low during the 3-week period prior to anthesis and was undetectable thereafter, although high levels of NR activity was found in the leaf and remained at detectable levels up to 4 weeks postanthesis (our unpublished data).

The question arises whether the *in vitro* activities accurately represent *in vivo* activities. Results from experiments with ^{15}N-labeled substrates showed that very little nitrate was reduced and assimilated in the stalk (Fig. 2). Ammonium was incorporated mainly into the amide group of glutamine, glutamate, and other amino acids, and to a minor extent into the amino group of glutamine (Fig. 2). The presence of MSX inhibited the conversion of ammonium into glutamine and other amino acids. These results are consistent with both the low *in vitro* activities of NR and the high *in vitro* activities of GS in maize stalks.

Ammonium produced by NR is regarded as the primary source of substrate for GS activity in maize, which as a C_4 plant has negligible photorespiration. The high activity of GS in the stalk raises the question of its function. A high activity of GS during leaf senescence was reported for *Sorghum vulgare* L. by Scott and Neyra (17). They suggested that GS may play a role in the assimilation of ammonium from sources other than nitrate reduction. Similar results were also reported by Thomas (23) and Storey and Beevers (19) in *Lolium temulentum* and *Pisum sativum,* respectively, suggesting that GS function may be related to proteolysis during senescence.

Developmental Changes in Stalk GS

Separation of GS isozymes showed that there are at least two forms of GS in the maize stalk: the predominant, putatively cytosolic GS1 form, and the minor, putatively plastidic GS2 form (6, 25). The kinetic properties of the GS isoforms were determined to establish optimum conditions for GS assays. The K_m values for Glu, ATP, and NH_2OH (Table II) were within the range of apparent K_m values listed for various plant species (13, 18). Although the pH optimum was similar for the two isozymes from both tissues, K_m values for all substrates were higher for GS2 compared with GS1 (Table II), as has been reported for other species (18). Both isozymes from the leaf had lower K_m values than those from stalk (Table II).

The developmental patterns of stalk GS isoform activities showed that, although GS1 activities increased from 3 weeks preanthesis to a maximum level at 2 weeks postanthesis and remained fairly constant thereafter, those of GS2 declined as the growing season progressed (Fig. 3). The ratios of GS1:GS2 activities were 6:1 at 3 weeks preanthesis, 11:1 at anthesis, 16:1 at 2 to 4 weeks postanthesis, and increased to 24:1 at 6 weeks postanthesis. When detached radish cotyledons were placed in the dark to induce senescence, mRNA for GS1 increased severalfold, whereas that for GS2 decreased rapidly (9). Study of the cell-specific expression of GS genes in trans-

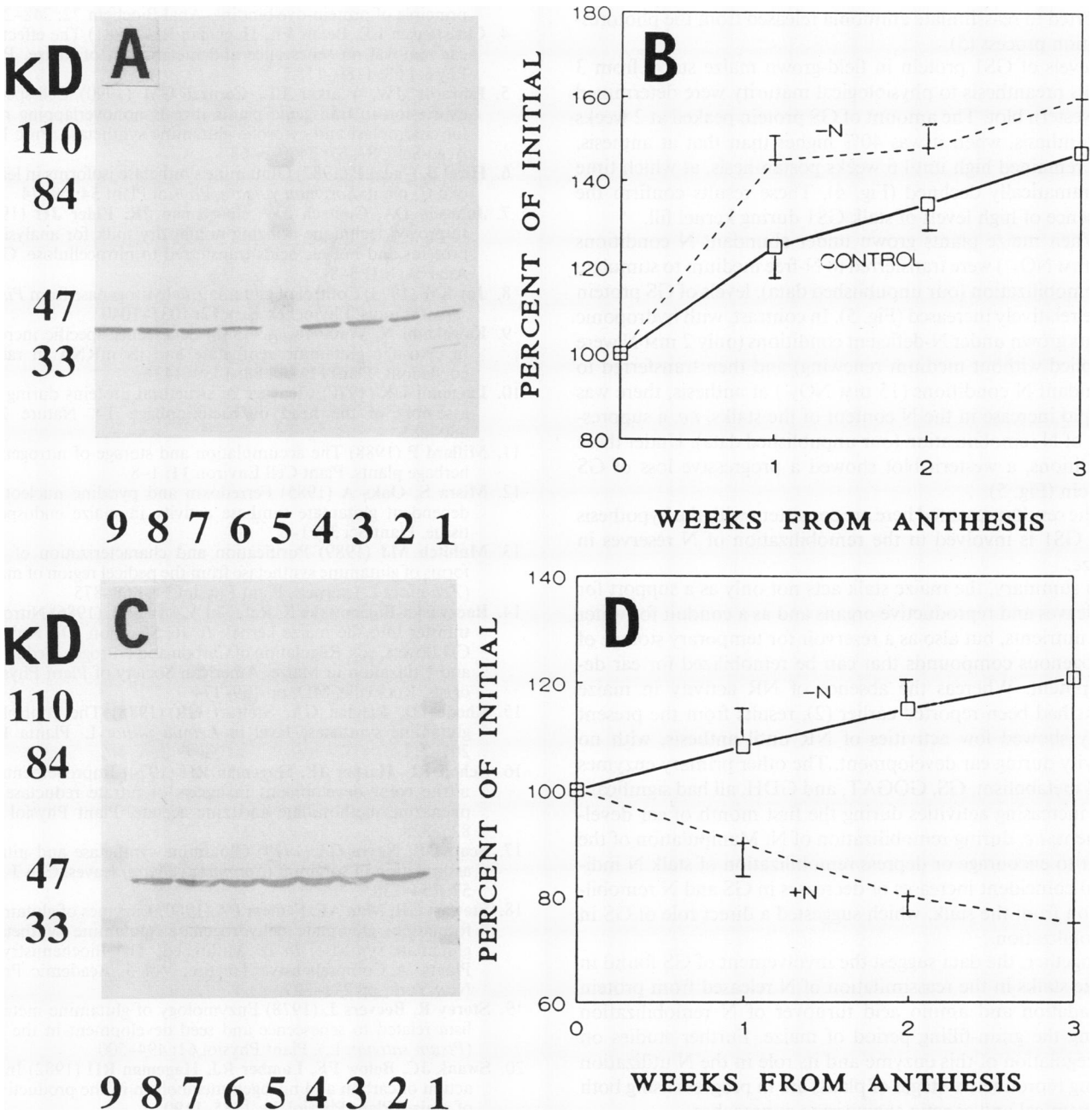

Figure 5. A, Western blot analysis of GS protein from stalk tissues of maize plants (B73 × LH51) grown under abundant N (15 mM NO_3^-, control) and transferred to N-free media (−N) at anthesis. Lanes 1 to 4, control plant harvested at weekly intervals starting from anthesis. Lane 5, molecular mass standards. Lanes 6 to 9, plants were transferred to N-free media. B, Gel scan analysis of blot as percentage of initial value (at anthesis). C, Western blot analysis of GS protein from stalk tissues of maize plants grown under N deficiency (−N) and transferred to N-abundant medium (15 mM NO_3^-, +N). Lanes 1 and 9, molecular mass standards. Lanes 2 to 5, plants grown under N deficiency. Lanes 6 to 8, plants were transferred to N-abundant medium at 1, 2, and 3 weeks from anthesis. D, Gel scan analysis of blot as percentage of initial value at anthesis.

genic tobacco has provided evidence that the cytosolic form, GS1, is confined to the phloem, where it has a role in the translocation of seed storage reserves during germination (5). GS2, which is present in chloroplasts of green tissue, was reported to reassimilate ammonia released from the photorespiration process (5).

Levels of GS1 protein in field-grown maize stalks from 3 weeks preanthesis to physiological maturity were determined by western blot. The amount of GS protein peaked at 2 weeks postanthesis, when it was 40% higher than that at anthesis, and remained high until 6 weeks postanthesis, at which time it dramatically declined (Fig. 4). These results confirm the presence of high levels of stalk GS1 during kernel fill.

When maize plants grown under abundant N conditions (15 mM NO_3^-) were transferred to N-free medium to stimulate N remobilization (our unpublished data), levels of GS protein were relatively increased (Fig. 5). In contrast, with hydroponic plants grown under N-deficient conditions (only 2 mM N were supplied without medium renewing) and then transferred to abundant N conditions (15 mM NO_3^-) at anthesis, there was a rapid increase in the N content of the stalks, *i.e.* a suppression of N remobilization (our unpublished data). Under these conditions, a western blot showed a progressive loss of GS protein (Fig. 5).

The results presented here are consistent with the hypothesis that GS1 is involved in the remobilization of N reserves in maize.

In summary, the maize stalk acts not only as a support for the leaves and reproductive organs and as a conduit for water and nutrients, but also as a reservoir for temporary storage of nitrogenous compounds that can be remobilized for ear development. Whereas the absence of NR activity in maize stalks had been reported earlier (2), results from the present study showed low activities of NR until anthesis, with no activity during ear development. The other primary enzymes of N metabolism, GS, GOGAT, and GDH, all had significant and increasing activities during the first month of ear development, *i.e.* during remobilization of N. Manipulation of the plant to encourage or depress remobilization of stalk N indicated coincident increases or decreases in GS and N remobilization from the stalk, which suggested a direct role of GS in remobilization.

Together, the data suggest the involvement of GS found in maize stalks in the reassimilation of N released from protein degradation and amino acid turnover of N remobilization during the grain-filling period of maize. Further studies on the regulation of this enzyme and its role in the N utilization during reproductive stages of plants are in progress using both biochemical and genetic engineering approaches.

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