Glutamine Synthetase and Ferredoxin-Dependent Glutamate Synthase Expression in the Maize (Zea mays) Root Primary Response to Nitrate

Evidence for an Organ-Specific Response

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To define further the early, or primary, events that occur in maize (Zea mays) seedlings exposed to NO3−, accumulation of chloroplast glutamine synthetase (GS2; EC 6.3.1.2) and ferredoxin-dependent glutamate synthase (Fd-GOGAT; EC 1.4.7.1), transcripts were examined in roots and leaves. In roots, NO3− treatment caused a rapid (within 30 min), transient, and cycloheximide-independent accumulation of GS2 and Fd-GOGAT transcripts. In addition, 10 μM external NO3− was sufficient to cause transcript accumulation. The induction was NO3− specific, since NH4Cl treatment did not affect mRNA levels. GS2 and Fd-GOGAT mRNA accumulation in roots was similar to that observed for nitrate reductase (NR) mRNA. Therefore, the four genes involved in NO3− assimilation (NR, nitrite reductase, GS2, and Fd-GOGAT) are expressed in the root primary response to NO3−, suggesting that all four genes can respond to the same signal transduction system. In contrast, relatively high levels of GS2 and Fd-GOGAT mRNAs were present in untreated leaf tissue, and NO3− treatment had little or no influence on transcript accumulation. Rapid, transient, and cycloheximide-independent NR mRNA expression was seen in the NO3−-treated leaves, demonstrating that NO3− was not limiting. The NO3−-independent constitutive expression of GS2 and Fd-GOGAT is likely due to the requirement for reassimilation of photorespiratory NH4+ in these young leaves.

When plant roots encounter NO3− in their environment, a series of complex physiological and biochemical events occur, including increased rates of NO3− transport and assimilation, metabolic activity, and root growth (Redinbaugh and Campbell, 1991). To characterize further this response at the molecular level, we are interested in defining the early, or primary, molecular changes that occur in roots exposed to NO3−. In maize (Zea mays), the rapid, transient, and protein synthesis-independent expression of transcripts for NR and NiR is among these primary events (Kramer et al., 1989; Privalle et al., 1989; Gowri et al., 1992). To define further this changing metabolic state, we have been interested in identifying other known and new genes that are similarly regulated in maize roots. Along with NR and NiR, the genes fundamentally involved with NO3− reduction, genes important in NO3− assimilation into amino acids, might also be expressed in the primary response.

GS (EC 6.3.1.2) and GOGAT (EC 1.4.7.1) are key enzymes for NO3− and NH4+ assimilation in higher plants (Oaks and Hirel, 1985; Lea et al., 1990). Multiple forms of these two enzymes are present in most higher plants, including maize. The cytosolic (GS1) and chloroplastic (GS2) forms of GS show distinct molecular weights and are encoded by differentially regulated genes in several species (Lightfoot et al., 1988; Coruzzi, 1991; Peterman and Goodman, 1991; Becker et al., 1992; Sakakibara et al., 1992). The two forms of GOGAT found in higher plants use different electron donors as substrates: one is a Fd-GOGAT and the other NAD(P)H-GOGAT (Oaks and Hirel, 1985; Lea et al., 1990). Light has little effect on the accumulation of GS1 proteins and transcripts, which are found at relatively high levels in nongreen tissues such as roots and etiolated shoots (Oaks and Hirel, 1985; Hayakawa et al., 1992). At least one of the GS1 genes is specifically expressed in the phloem, where it may be important in N translocation (Edwards et al., 1990). NAD(P)H:GOGAT activity is largely localized to the plastids of nongreen tissues, where expression is not affected by light (Oaks and Hirel, 1985; Hayakawa et al., 1992). In green leaves, the chloroplast-localized GS2 and Fd-GOGAT are important in the reassimilation of NH4+ derived from photorespiration, in the assimilation of NH4+ derived from NO3− reduction, and in amino acid catabolism (Lea et al., 1990; Coruzzi, 1991). As expected with genes that function in photorespiration, the GS2 and Fd-GOGAT genes are light regulated, and the mRNAs are present at very low levels.

Abbreviations: Cat1, catalasel; CHX, cycloheximide; Fd-GOGAT, ferredoxin-dependent GOGAT; GOGAT, glutamate synthase; GS, glutamine synthetase; GS1, cytosolic GS; GS2, plastid GS; NiR, nitrite reductase; NR, nitrate reductase.
in etiolated leaves from several plants (Cock et al., 1991; Peterman and Goodman, 1991; Becker et al., 1992; Kozaki et al., 1992; Sakakibara et al., 1992; Zehnacker et al., 1992). In nongreen tissues, GS2 and Fd-GOGAT are found in plastids, along with NiR (Oaks and Hirel, 1985; Lea et al., 1990). The transcripts accumulate during greening to become relatively abundant in green leaf tissue. GS2 and Fd-GOGAT proteins are implicated in NO₃⁻ assimilation through the ability of isolated chloroplasts and root plastids to assimilate NO₃⁻ into Glu and Gln (Lea et al., 1990). External N has been found to affect GS and GOGAT expression in several higher plants. For example, GS and Fd-GOGAT activity is slowly increased by external NO₃⁻ or NH₄⁺ in maize (Sugiharto and Sugiyama, 1991) and Kalanchoe (Santos and Salema, 1992). In addition, external NO₃⁻ caused increases in GS2 and Fd-GOGAT protein accumulation over several days in cultured rice cells (Hayakawa et al., 1992). Most recently, Kozaki et al. (1992) showed that the rice GS2 promoter responds to NH₄⁺ and NO₃⁻ over several hours in transformed tobacco plants.

It was previously shown in maize and other higher plants that both GS2 (Lightfoot et al., 1988; Peterman and Goodman, 1991; Becker et al., 1992; Sakakibara et al., 1992), and Fd-GOGAT (Sakakibara et al., 1992; Zehnacker et al., 1992) transcripts were present at low levels in root tissues. However, it is not known how N nutrition influences the accumulation of these transcripts in maize roots. Although some effects of N compounds on leaf GS2 and Fd-GOGAT have been described, little is known about the immediate response of these genes to environmental NO₃⁻ in green tissues. Therefore, we examined the effect of NO₃⁻ on GS2 and Fd-GOGAT transcript accumulation during the primary response to NO₃⁻.

MATERIALS AND METHODS

Plant Material and Treatments

Corn seed (Zea mays L. var W64A × W182E) was germinated for 3 d in darkness at 30°C on paper moistened with 0.1 mM CaSO₄. Subsequently, the seedlings were transferred to aerated hydroponic culture on 0.1 mM CaSO₄ with 16/8 h light/dark cycle at 32/27°C for 2 d prior to initiation of treatment (Gowri et al., 1992). For the CHX and NO₃⁻ treatments, the hydroponic solution was brought to 50 μg/mL of CHX beginning approximately 4 h into the light period. One hour later, the solution was brought to 10 mM KNO₃ for an additional 2 h. To test the effect of time of NO₃⁻ treatment on the accumulation of RNA, the hydroponic solution was brought to 10 mM KNO₃ 2 h into the light period, and seedlings were harvested at the times indicated. For analysis of the effect of low external NO₃⁻ on transcript accumulation, KNO₃ was added at the indicated concentration for a 2-h period. Following treatment, roots and leaves were frozen in liquid N₂ and stored at −70°C. All treatments were repeated at least twice, and representative results are shown.

RNA Blot Analysis

Total RNA was isolated, separated on denaturing gels, transferred to nitrocellulose, and hybridized with radiolabeled cDNA as described (Redinbaugh et al., 1988). Nonspecifically bound probe was removed by washing the filters to a stringency of 0.1 × SSC (15 mM Na-citrate, pH 7.0, 15 mM NaCl, 0.1% SDS) at 65°C for at least 1 h. Insert cDNAs were isolated from cDNA clones for the maize chloroplastic GS2 (MGS1; Snustad et al., 1988), maize Fd-GOGAT (pFDGGT11; Sakakibara et al., 1991), or Cat1 (pCat1.1; Redinbaugh et al., 1988). For the histograms, the autoradiograms shown in the figures were scanned with an LKB UltraScan XL laser densitometer interfaced with GelScan XL Software (LKB) to determine the intensity of the hybridization signal. Blots of the same RNA probed with NR (pZmnr1; Gowri and Campbell, 1989) were also scanned, and the data are included for comparison. Because these, or very similar, analyses have been published (Gowri and Campbell, 1989; Long et al., 1991; Gowri et al., 1992), photographs of the NR northern blots are not shown.

RESULTS AND DISCUSSION

Three criteria have been used to indicate that a gene is expressed in the primary response to NO₃⁻: CHX-independent NO₃⁻ induction of transcript accumulation; rapid and transient transcript accumulation; and induction of transcript accumulation by low external NO₃⁻ concentrations (Redinbaugh and Campbell, 1991; Gowri et al., 1992). To test whether GS2 and Fd-GOGAT are expressed in the maize root primary response to NO₃⁻, cDNAs encoding the maize GS2 (Snustad et al., 1988) and Fd-GOGAT (Sakakibara et al., 1991) were hybridized with maize RNA blots at high stringency. Under similar conditions, Southern blot analysis of maize genomic DNA indicated that there is likely to be a single copy of the Fd-GOGAT gene (Sakakibara et al., 1991). Under the conditions used in these experiments, the Fd-GOGAT cDNA hybridized with an RNA species of approximately 5.6 kb, as was previously observed (Sakakibara et al., 1991, 1992). The GS2 cDNA (MGS1) was originally isolated by complementation of an Escherichia coli GS mutant, and an essentially identical cDNA (pGS202) was isolated by Sakakibara et al. (1992). In contrast with four other GS cDNAs, the GS2 cDNA encodes 10 extra amino acids at the NH₂ terminus and hybridizes with a light-regulated transcript in maize leaves (Sakakibara et al., 1992). These properties are characteristic of GS2. Under less stringent conditions than those used here, little or no cross-hybridization of this cDNA with GS1 could be detected (Sakakibara et al., 1992). In addition, a 200-bp EcoRI-NcoI fragment corresponding to the 3' untranslated region of the GS2 mRNA hybridized with the RNA blots in the same pattern as the full-length cDNA (data not shown).

Initially, the effect of a 2-h, 10-mM NO₃⁻ treatment in the presence and absence of CHX on GS2 and Fd-GOGAT mRNA levels was tested (Fig. 1). As was previously shown (Sakakibara et al., 1992), both transcripts are present at low levels in NO₃⁻-starved seedlings (Fig. 1A). The transcript level increased dramatically in seedlings exposed to NO₃⁻, and CHX did not prevent transcript accumulation. For reference, blots were probed with a gene whose expression in roots is not affected by NO₃⁻ (Cat1, Gowri et al., 1992). Relative to Cat1, the pattern of expression observed for GS2 and Fd-GOGAT (Fig. 1B) was similar to that previously shown for NR (Gowri et al., 1992). CHX treatment alone
caused a slight increase in transcript level, whereas \(\text{NO}_3^-\) treatment gave approximately a 5-fold increase. For NR and GS2, treatment with both CHX and \(\text{NO}_3^-\) resulted in further increased levels of mRNA, but CHX had no effect on the \(\text{NO}_3^-\) induction of Fd-GOGAT. The level of CHX used in these experiments was sufficient to prevent the accumulation of detectable NR activity in maize roots and reduced cytoplasmic protein synthesis by about 85% (Gowri et al., 1992). The data are consistent with the protein synthesis-independent accumulation of GS2 and Fd-GOGAT mRNA, although a requirement for residual protein synthesis or NR activity cannot be excluded. Protein synthesis inhibitors have been found to cause the accumulation of NR (Gowri et al., 1992), the mammalian primary response genes \(\text{fos}\) and \(\text{myc}\) (Atwater et al., 1990), and three auxin-induced transcripts (Key, 1989), among others. For these highly regulated genes, it is likely that the inhibition of translation prevents mRNA turnover (Atwater et al., 1990).

When maize roots were exposed to \(\text{NO}_3^-\) for various lengths of time, a rapid and transient increase in expression of GS2 and Fd-GOGAT mRNAs was seen (Fig. 2). Within 30 min after \(\text{NO}_3^-\) addition, an increased transcript level relative to \(\text{Cat}1\) could be detected (Fig. 2, A and B). The mRNA levels continued to increase for 1 to 2 h of \(\text{NO}_3^-\) exposure, and declined substantially by 8 to 16 h. This pattern of \(\text{NO}_3^-\)-induced expression is nearly identical to that found for NR (Fig. 2B; Gowri and Campbell, 1989) and NiR in root tissue (Kramer et al., 1989). In the blot shown, the level of \(\text{Cat}1\) mRNA appeared to be slightly low in the 0 time sample (Fig. 2A), although \(\text{NO}_3^-\) treatment did not influence subsequent \(\text{Cat}1\) transcript levels. Nevertheless, little GS2 or Fd-GOGAT mRNA was detected in untreated tissues (cf. Fig. 1A and Fig. 3A), and these transcripts subsequently accumulated to a
much greater extent than the control. Thus, root GS2 and Fd-GOGAT mRNAs showed the same rapid and transient response to external NO$_3^-$ as the other primary response genes, NR and NiR.

To determine whether GS2 and Fd-GOGAT mRNAs accumulated in response to very low levels of external NO$_3^-$, maize seedlings were exposed to various NO$_3^-$ concentrations for 2 h, and the levels of GS2 and Fd-GOGAT transcript were determined (Fig. 3). Both mRNAs accumulated in response to 10 mM KNO$_3$ (Fig. 3A). The mRNA levels were about 5-fold higher than the control between 10 mM and 1 mM NO$_3^-$ for both transcripts, and a somewhat higher level of Fd-GOGAT mRNA was found in response to 10 mM NO$_3^-$ (Fig. 3B). A similar response was observed for NR (Gowri et al., 1992; Fig. 3B). For NR, an increased response to high external NO$_3^-$ concentrations was attributed either to increased expression in a greater number of cell types, as occurred with NR protein (Rufty et al., 1986), or to expression of multiple genes (Gowri et al., 1992). In contrast with NR, however, previous results indicated a single gene for both GS2 and Fd-GOGAT (Snustad et al., 1988; Sakakibara et al., 1991, 1992).

The data presented above indicate that both GS2 and Fd-GOGAT are expressed in the maize root primary response to NO$_3^-$. Both mRNAs accumulate rapidly and transiently in roots exposed to NO$_3^-$. This response occurs even at very low external concentrations of NO$_3^-$. Importantly, the accumulation of transcript appears to be independent of protein synthesis. The tight coupling of NR/NiR expression with that of GS2/Fd-GOGAT in roots is not surprising, since the assimilation of reduced NO$_3^-$ in roots is considered to occur in plastids (Lea et al., 1990). Although the effect of NO$_3^-$ on the abundance of GS2 and Fd-GOGAT proteins in roots has not been examined, the levels of GS2 and Fd-GOGAT proteins in cultured rice cells increased in response to NO$_3^-$ (Hayakawa et al., 1990). In these cells, neither GS2 nor Fd-GOGAT protein levels responded to a similar NH$_4^+$ treatment. To test the effect of N on GS2 and Fd-GOGAT expression, maize seedlings were given a 2-h treatment with 10 mM NH$_4$Cl. Neither GS2 nor Fd-GOGAT transcript accumulated in NH$_4^+$-treated roots (data not shown). The data are consistent with the idea that NH$_4^+$ uptake by roots is assimilated via cytoplasmic and possibly vascular GS1 and NADH-GOGAT, prior to translocation of assimilates to the shoot (Edwards et al., 1990; Hayakawa et al., 1990; Lea et al., 1990).

Although the accumulation of the GS2 and Fd-GOGAT transcripts appeared to respond to external NO$_3^-$, it was possible that either the K$^+$ counter-ion or exposure of the roots to light during treatment was responsible for the observed effects. However, neither transcript accumulated in response to treatments with 50 mM KCl or 1 mM K-phosphate (data not shown). No particular effort was made to exclude light from the roots in hydroponic culture. In addition, no accumulation of the transcripts was observed in untreated roots, which were exposed to light similarly to the NO$_3^-$-treated roots (data not shown). Thus, root GS2 and Fd-GOGAT expression appears to respond specifically to NO$_3^-$. For NR, NO$_3^-$ was shown to be the active species for the induction of gene expression in several systems (Aslam and Huffaker, 1989; Redinbaugh and Campbell, 1991).

Both NR and NiR transcripts are expressed in the maize leaf primary response to NO$_3^-$. (Kramer et al., 1989; Gowri et al., 1992). In all tissues, NR and NiR are considered to function only in NO$_3^-$ reduction. As mentioned previously, because GS2 and Fd-GOGAT have multiple functions in the chloroplast (Lea et al., 1990), differences in the organ response of these two genes to NO$_3^-$ might be expected. To determine whether GS2 and Fd-GOGAT are expressed in the primary response of maize leaves to NO$_3^-$, leaf tissue isolated from plants, treated as above, was tested for levels of GS2 and Fd-GOGAT transcripts.

As has been previously reported (Sakakibara et al., 1992), relatively high levels of the GS2 and Fd-GOGAT transcripts were found in green leaves not treated with NO$_3^-$ (Fig. 4). A 2-h treatment with 10 mM NO$_3^-$ had no effect on GS2

![Figure 3.](image-url)

**Figure 3.** The induction of GS2 and Fd-GOGAT RNA accumulation in response to low levels of external NO$_3^-$ in maize roots. Seedlings were treated with the indicated concentration of KNO$_3$ for 2 h. A, Northern blots of root total RNA (10 μg) were probed with GS2, Fd-GOGAT, or Cat1 cDNA as described in "Materials and Methods." The data presented are from single blots; however, in the photograph the lanes are rearranged for visual clarity. B, The relative induction for NR, GS2, and Fd-GOGAT was determined as for Figure 1B.
Induction of Glutamine Synthetase and Fd-Dependent Glutamate Synthase

Treatment

Figure 4. The effect of NO$_3^-$ and CHX on the accumulation of GS2 and Fd-GOGAT transcripts in maize leaves. Five-day-old maize seedlings were pretreated for 1 h with 50 μg/mL of CHX, followed by a 2-h treatment with 10 mM KNO$_3$, as indicated above the figure. A, Northern blots of total RNA (10 μg) were probed with GS2, Fd-GOGAT, or Cat1 cDNA as described in “Materials and Methods.” B, The relative induction for NR, GS2, and Fd-GOGAT was determined as for Figure 1B.

transcript accumulation and resulted in a small increase in Fd-GOGAT mRNA (Fig. 4A). Importantly, when CHX was present, NO$_3^-$ treatment had no effect on the level of GS2 or Fd-GOGAT transcript. Over several different experiments, NO$_3^-$ treatment either had no effect, or had a small positive effect on GS2 and Fd-GOGAT mRNA levels in leaves. In contrast, NR mRNA accumulated in leaves with the typical primary response pattern (Fig. 4B; Gowri et al., 1992). Here, NO$_3^-$ treatment resulted in a 5-fold increase in transcript level, compared with a 1- to 2-fold increase in GS2 and Fd-GOGAT mRNA. In maize seedlings treated with NO$_3^-$ for varying lengths of time, leaves accumulate slightly higher levels of GS2 and Fd-GOGAT transcript (Fig. 5). Whereas NR transcript accumulated rapidly and transiently to about 4-fold higher levels after 2 h of NO$_3^-$ treatment, the levels of GS2 and Fd-GOGAT mRNAs varied less than 2-fold over the 6-h experiment. Although slow delivery of NO$_3^-$ from the hydroponic solution to the leaf could be a problem, it was recently shown that in NO$_3^-$-grown maize seedlings, delivery of [${}^{15}$N]NO$_3^-$ to shoots occurs within minutes of exposure (Lazof et al., 1992). Also, NR mRNA accumulated as expected in each case, demonstrating that the NO$_3^-$ and CHX treatments were effective. Thus, GS2 and Fd-GOGAT appear to respond to environmental NO$_3^-$ in an organ-specific manner.

Unlike NR, GS2 and Fd-GOGAT transcripts either are not expressed in the leaf NO$_3^-$ primary response or the response is so quantitatively small as to make it difficult to discern from variation in the background levels of expression. A somewhat elevated level of GS2 and Fd-GOGAT mRNAs in NO$_3^-$-treated leaves might be consistent with the observed increase in the activity of the rice plastidic GS2 promoter in response to NO$_3^-$ and NH$_4^+$ over a 2- to 5-h period (Kozaki et al., 1992). In addition, although increased total GS activity could not be detected, a small increase in Fd-GOGAT activity was found over 2 to 3 d in NO$_3^-$-treated 2-week-old maize leaves (Sugiharto and Sugiyama, 1992). In this case, more pronounced increases in GS2 and Fd-GOGAT occurred in NH$_4^+$-treated leaves, and the increased activity paralleled increases in phosphoenolpyruvate carboxylase expression. Together, the data suggest that NO$_3^-$ does not act as the

Figure 5. Time course of the NO$_3^-$ induction of GS2 and Fd-GOGAT mRNA expression in maize leaves. Maize seedlings were treated with 10 mM KNO$_3$ for the indicated times, and leaf tissue was harvested. A, Northern blots of total RNA (10 μg) were probed with GS2, Fd-GOGAT, or Cat1 cDNA as described in “Materials and Methods.” B, The relative induction for NR, GS2, and Fd-
primary positive control for GS2 and Fd-GOGAT expression in leaves, but exerts an indirect effect as an N compound.

One reason for the differential NO\textsubscript{3}- regulation of GS2 and Fd-GOGAT between roots and leaves is that the leaf enzymes also function in the reassimilation of photosynthetic N\textsubscript{2+}, which can occur at 10 times the rate of NO\textsubscript{3}- assimilation in a C\textsubscript{3} plant (Givan et al., 1988). The seedlings used in this study were very young, having only an expanded first and partially expanded second leaf. At this developmental stage, both phosphoenolpyruvate carboxylase and NADP+–malic enzyme activities are about 10% of those found in the second and fourth leaves (Yamaya and Oaks, 1988). Because photosynthesis is probably largely C\textsubscript{3} in character at this developmental stage (Nelson and Langdale, 1992), the expression of GS2 and Fd-GOGAT transcripts in NO\textsubscript{3}-starved green leaves is not surprising. This is clearly different than the regulation of NR and NiR in leaves, where both enzymes function in a single pathway and are expressed in the primary response to NO\textsubscript{3}-, much as they are in roots and cultured cells (Kramer et al., 1989; Privatelle et al., 1989; Gowri et al., 1992).

The experiments presented here are based on NO\textsubscript{3}- induced increases in steady-state levels of transcripts for the NO\textsubscript{3} assimilation genes. This NO\textsubscript{3} induction has a large transcriptional component for NR and NiR (Melzer et al., 1989; Lu et al., 1990; Back et al., 1991; Redinbaugh and Campbell, 1991). The fact that GS2 and Fd-GOGAT transcripts are expressed in the root primary response to NO\textsubscript{3} suggests that they too are transcribed in response to NO\textsubscript{3}- and could contain NO\textsubscript{3}–response elements similar to those considered to be present on NR and NiR. Although these elements have yet to be identified on higher plant NR, NO\textsubscript{3}– regulation was localized to a 3-kb region of the spinach NiR gene (Back et al., 1991). Given the similarity of the effect of environmental NO\textsubscript{3}– on NR and NiR expression in roots and leaves, both tissues would appear to have similar signaling systems for NO\textsubscript{3}– induction. If this is the case, then GS2 and Fd-GOGAT expression can occur by at least one additional mechanism in leaves.

In conclusion, the regulation of maize GS2 and Fd-GOGAT expression is organ specific. Like NR and NiR, GS2 and Fd-GOGAT are expressed in the maize root primary response to NO\textsubscript{3}. Both transcripts accumulate rapidly and transiently in seedlings exposed to environmental NO\textsubscript{3}–. The response occurs at very low external NO\textsubscript{3}– concentrations, is specific for NO\textsubscript{3}, and is apparently independent of cytoplasmic protein synthesis. Although NR and NiR are similarly regulated in maize leaves and roots, there is no clear short-term effect of NO\textsubscript{3}– on GS2 and Fd-GOGAT transcript accumulation in green leaves. At least in part this is because both transcripts are relatively abundant in untreated green leaves, presumably for the reassimilation of N\textsubscript{2+} released during photosorption.

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