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Regulation of the Accumulation and Reduction of Nitrate by Nitrogen and Carbon Metabolites in Maize Seedlings[1]

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The accumulation and reduction of nitrate in the presence of the nitrogen metabolites asparagine (Asn) and glutamine (Gln) and the carbon metabolite sucrose (Suc) were examined in maize (*Zea mays* L.) seedlings in an attempt to separate their effects on the nitrate uptake system and the nitrate reduction system. After 8 h of exposure to nitrate in the presence of 1 mM Asn, tissue nitrate accumulation was reduced at 250 μM external nitrate, but not at 5 mM Asn. The induction of nitrate reductase (NR) activity was reduced at both external nitrate concentrations. In the presence of 1 mM Gln or 1% Suc, tissue nitrate concentration was not significantly altered, but the induction of root NR activity was reduced or enhanced, respectively. The induction of root nitrite reductase (NiR) activity was also reduced in the presence of Asn or Gln and enhanced in the presence of Suc. Transcript levels of NR and NiR in roots were reduced in the presence of the amides and enhanced in the presence of Suc. When Suc was present in combination with either amide, there was complete relief from the inhibition of NiR transcription observed in the presence of amide alone. In the case of NR, however, this relief from inhibition was negligible. The inhibition of the induction of NR and NiR activities in the presence of Gln and Asn is a direct effect and is not the result of altered nitrate uptake in the presence of these metabolites.

Nitrate, the primary nitrogen source for most land plants, is absorbed by a specific nitrate uptake permease(s) in plant roots, reduced to nitrite by NR in the cytoplasm, and then reduced to ammonium by NiR in the plastid. Therefore, nitrate uptake permease(s), NR, and NiR constitute the first three enzymes of the nitrate assimilatory pathway and are subject to regulation by several endogenous and environmental stimuli, including nitrate, Gln, light, and Suc (for review, see Crawford and Arst, 1993; Oaks, 1994; Sivasankar and Oaks, 1996). Nitrate induces the expression of both the uptake and reduction systems, whereas Suc and light enhance this induction. Gln represses the induction of the nitrate assimilatory system. The regulation of NR by nitrate, Suc, and Gln occurs at the transcriptional level, whereas the regulation by light occurs at both the transcriptional and posttranslational levels (Melzer et al., 1989; Cheng et al., 1992; Vincentz et al., 1993; Li et al., 1995; MacKintosh et al., 1995). Because the effectors Gln and Suc influence both the uptake and reduction systems, and the presence of nitrate is required for the induction of NR and NiR, it is possible that any effect of Gln and Suc on NR and NiR is an indirect effect arising out of a direct effect on nitrate uptake. Separation of the effects of these metabolites on the uptake and reduction systems has not yet been attempted.

Nitrate uptake is mediated by two or more classes of nitrate transporters, referred to as the low- and high-affinity classes, which are present at the plasmalemma of root cells. In barley at least three transport systems are known to operate (Glass and Siddiqi, 1995). One belongs to the low-affinity class, operates at high external nitrate concentrations, and is not substrate-inducible but is subject to negative feedback by end products. The other two belong to the high-affinity class and operate at low external nitrate concentrations. One of these is substrate-inducible and is also repressible by end products, whereas the other is constitutive. Regulatory studies of nitrate uptake have focused mainly on the inducible, high-affinity transport system and therefore have used less than 250 μM nitrate. However, experiments in which the regulatory effects of amino acids on nitrate reduction were studied have invariably been done with greater than 5 mM nitrate. Therefore, the results of experiments on nitrate uptake and those on nitrate reduction cannot be directly compared to analyze the separate effects of regulatory factors on these two phenomena.

Although the regulatory effect of Gln on NR has been examined extensively in both fungal and plant systems, its effect on NiR and nitrate uptake has not been examined in detail (Marzluf, 1981; Vincentz et al., 1993; Li et al., 1995). Furthermore, the regulatory effect of the other amide, Asn, on NR has not been analyzed, even though it has been implicated in the inhibition of nitrate uptake (Lee et al., 1992; Müller and Touraine, 1992). Our previous results showed that Asn has a similar or even greater inhibitory effect on the induction of NR than that produced by Gln (Sivasankar and Oaks, 1995).

In this paper we characterize the regulation of the induction of nitrate uptake, NRA, and NiRA by Asn, Gln, and Suc at two external NO_3^- concentrations, 250 μM and 5 mM, to separate their individual effects on uptake and

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Abbreviations: NiR, nitrite reductase; NiRA, nitrite reductase activity; NR, nitrate reductase; NRA, nitrate reductase activity; OPA, *o*-phthalaldehyde.

reduction in maize (*Zea mays*) seedlings. We also determined the effect of these metabolites on the transcription of the NR and NiR gene. Finally, since Suc is known to inhibit the expression of Asn synthetase (Stulen and Oaks, 1977; Genix et al., 1994; Lam et al., 1994), we also attempted to determine whether the up-regulation of NR and NiR expression produced by Suc is an indirect effect resulting from a reduction in tissue Asn levels.

MATERIALS AND METHODS

Seed Germination and Plant Growth

Kernels of maize (*Zea mays* L. cv Pioneer hybrid no. 3475) were soaked for 5 to 6 h and then allowed to germinate in the dark on paper towels soaked in a modified one-tenth-strength Hoagland solution that contained no combined nitrogen. After 48 h the seedlings were transferred to a hydroponic system containing the same nutrient solution (for description, see Sivasankar and Oaks, 1995). Seedlings were grown in growth chambers maintained at 28/26°C day/night temperatures, with a 16-h light/8-h dark cycle and a light intensity of 225 μmol m^{-2} s^{-1} at canopy level. Asn (1 mM), Gln (1 mM), or Suc (1%) was added to the hydroponic solution 5 or 6 d after imbibition. Nitrate as 250 μM or 5 mM KNO_3 was added to the hydroponic solution 2 h after the amide or Suc additions. Control plants received nitrate alone. Streptomycin sulfate (250 μg mL^{-1}), penicillin (K^+ salt; 10 μg mL^{-1}), and chloramphenicol (10 μg mL^{-1}) were added to the growth medium of both control and treated plants at the same time amides or Suc were added. Plants were harvested for root and shoot or for xylem sap 8 or 20 h after induction with nitrate. Xylem sap was collected by excising shoots approximately 0.5 cm above the first node and collecting with a Pasteur pipette. Shoots above the second node and roots below the first node were harvested, frozen in liquid nitrogen, ground to a fine powder, and stored at −70°C. Tissues were extracted the day after each harvest for determination of enzyme activity and protein.

Tissue Amide Concentration

Amides in tissue extracts were separated and quantified using HPLC (model 421 chromatograph, Beckman) and a fluorometer (Gilson, Middleton, WI). Frozen tissue powder (250 mg) was ground in 1 mL of 80% ethanol and centrifuged at 13,000 rpm for 2 min in an Eppendorf tabletop centrifuge. The supernatant was filtered through a 0.45-μm filter (HV, Millipore). A 25-μL aliquot of appropriately diluted filtrate was mixed with 25 μL of OPA (*o*-phthalaldehyde, excitation at 360 nm and emission at 455 nm) with simultaneous activation of an HPLC program that allowed the isocratic separation of four amino acids and two amides. A reverse-phase column (Ultrasphere, ODS, Beckman) with 5-μm particles and dimensions of 4.6 mm × 25 cm was used. The amino acids derivatized with OPA were separated according to the method described by Winspear and Oaks (1983). Buffer A consisted of 0.02 M sodium phosphate, pH 6.8, and 30% methanol, and buffer B was pure methanol. The buffers were filtered and degassed prior to use. The OPA reagent was prepared by dissolving 50 mg of OPA in 1 mL of methanol, to which 9 mL of 0.4 M sodium borate buffer, pH 9.4, and 200 μL of 2-mercaptoethanol were added.

Tissue Nitrate Concentration

For nitrate determination the frozen powder of shoot or root was extracted using boiling water, in the ratio of 250 mg of powder to 10 mL of water. The extraction period was 30 min, after which the extracts were filtered through filter paper (Whatman no. 1). After the residue was washed with boiling water, the volume of the filtrate was brought to 10 mL. The sample was again filtered through a 0.45-μm filter prior to nitrate determination.

Nitrate was analyzed on the HPLC system using an anion chromatography column (catalog no. 3001C405, VYDAC, Hesperia, CA). The buffer system consisted of 2 mM phthalic acid, adjusted to pH 5.5 with KOH. A flow rate of 1.5 mL/min was used for 5 min. An aliquot of 20 μL of the sample or standard was injected onto the HPLC column. Standards were linear in the range of 1 to 5 mM KNO_3 concentration. Nitrate was detected using vacancy UV light at 280 nm with reversed leads to the integrator (Walker et al., 1989). Tissue nitrate concentration is expressed as micromoles per gram fresh weight.

Nitrate Concentration of Xylem Sap

The xylem sap collected during a 1-h period was measured for volume and then centrifuged in an Eppendorf tabletop centrifuge at 13,000 rpm for 2 min to remove any particulate matter. The supernatant was filtered through a 0.45-μm filter prior to analytical determinations. Nitrate in the sap was determined colorimetrically by nitration of salicylic acid as described by Cataldo et al. (1975).

Assay of NRA and NiRA

Tissue samples were extracted for NRA and NiRA assays, and NRA in extracts was determined as previously described (Sivasankar and Oaks, 1995). The NiRA in the extract was assayed using the method described by Wray and Filner (1970), with slight modifications. The assay mixture consisted of 75 mM Tris-HCl, pH 8.0, 5 mM methyl viologen dissolved in H_2O, 50 mM sodium dithionite (prepared fresh in a solution of 30 mM sodium bicarbonate dissolved in H_2O), and 1 mM sodium nitrite. The reaction was initiated by the addition of freshly prepared sodium dithionite solution, and the assay mixture was incubated at 25°C for 20 min. At the end of the incubation period the mixture was vortexed at top speed to oxidize the remaining dithionite, and an aliquot of 0.1 mL was diluted to 1 mL with H_2O. To this was added 1 mL of 1% (w/v) sulfanilamide prepared in 1 N HCl and 1 mL of 0.01% (w/v) *N*-1-naphthylethylene-diamine-dihydrochloride prepared in H_2O. The resultant color was measured 30 min later on a spectrophotometer at 540 nm. NiRA is expressed as micromoles of nitrite consumed per hour per gram fresh weight.

RNA Isolation and Hybridization

Total RNA was extracted from 1 g of frozen powder using the protocol described by Chang et al. (1993). The RNA was quantified spectrophotometrically and uniform loading was ensured based on ethidium bromide staining of the rRNA. For RNA-blot hybridization, 10 μg of total RNA was denatured by incubating at 60°C for 5 min in 50% formamide, 7% formaldehyde, and 25 mM Mops (pH 7.0) and size-fractionated by electrophoresis through a 1.5% agarose gel that contained 50 mM Mops (pH 7.0) and 2.2 M formaldehyde. The RNA was blotted onto nitrocellulose membranes by capillary action (Sambrook et al., 1989). The probe used for hybridization was either an NiR cDNA insert from the plasmid pCIB808 (Lahners et al., 1988) or an NR cDNA insert from the plasmid pCIB 1501 (Long et al., 1992). The fragment was radiolabeled with [α-^{32}P]dCTP (Amersham) by random priming to a specific activity of 1×10^8 cpm μg^{-1}. The filter was prehybridized and hybridized according to the procedure described by Sambrook et al. (1989) and then washed, dried, and subjected to autoradiography on XAR film (Kodak).

Statistical Analysis

Data from experiments repeated at least three times were subjected to statistical analysis. Experiments repeated at different times were considered replicates or blocks in a completely randomized block design. The minimum sample size was 30 plants pooled together. Data were analyzed by one-way analysis of variance using SYSTAT (SYSTAT Inc., Evanston, IL). Where F values indicated significance ($P < 0.05$), individual means were compared using Student's *t* test.

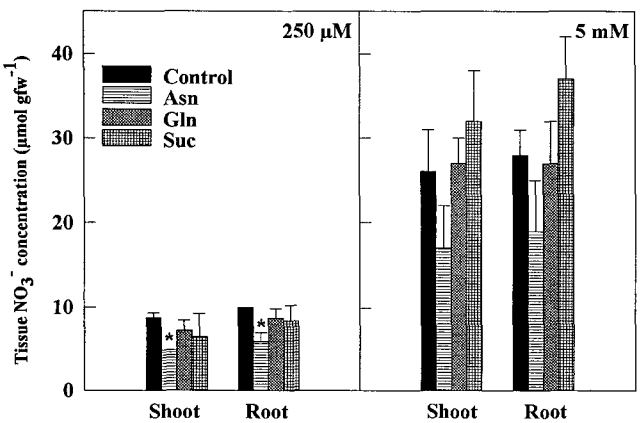

Figure 1. Effect of 1 mM Asn, 1 mM Gln, or 1% Suc on nitrate concentration in shoots and roots of 6-d-old maize seedlings induced with 250 μM or 5 mM KNO_3. Seedlings were harvested 8 h after induction with nitrate. Values represent means ± SD of three separate experiments. gfw, Grams fresh weight; *, significant difference ($P < 0.05$) between amide-/Suc-treated plants and untreated controls.

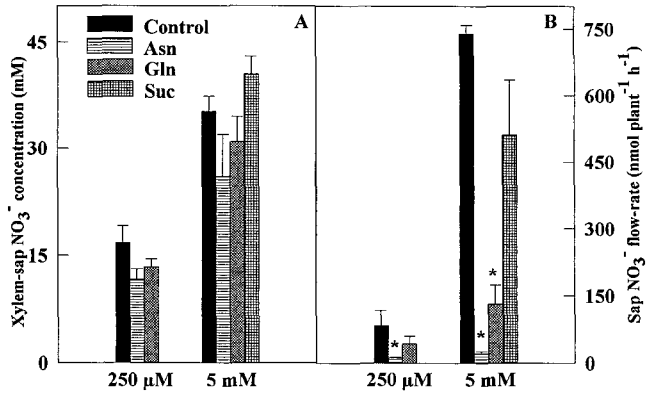

Figure 2. Effect of 1 mM Asn, 1 mM Gln, or 1% Suc on nitrate concentration of xylem sap (A) and xylem sap nitrate flow rate (B) in detopped 6-d-old maize seedlings induced with 250 μM or 5 mM KNO_3. Values represent means ± SD of three separate experiments. *, Significant difference ($P < 0.05$) between amide-/Suc-treated plants and untreated controls.

RESULTS

Effect of Amides and Suc on Tissue Nitrate Accumulation

The nitrate accumulation in shoots and roots of 6-d-old maize seedlings was determined after 8 h of exposure to 250 μM or 5 mM nitrate in the presence of 1 mM Asn, 1 mM Gln, or 1% Suc. Shoot and root nitrate concentrations in the absence of the exogenous effectors were 8.7 and 10.0 μmol g^{-1} fresh weight, respectively, at 250 μmol external nitrate, and 26.0 and 28.0 μmol g^{-1} fresh weight, respectively, at 5 mM external nitrate (Fig. 1). The presence of Asn significantly reduced nitrate accumulation in both shoot and root to approximately 60% of that in the untreated controls when the external nitrate was 250 μM. At 5 mM external nitrate, however, Asn did not produce a significant reduction in shoot or root nitrate accumulation. The effect of Gln and Suc on tissue nitrate accumulation was not significant at either external nitrate concentration.

The concentration of nitrate in the xylem sap was also determined to assess the effect of amides and Suc on nitrate translocation from root to shoot. In the absence of exogenous effectors, the concentration of nitrate in the exudate at 5 mM external nitrate was twice that at 250 μM external nitrate (Fig. 2A). The presence of amides and Suc did not significantly alter the concentration of nitrate in the exudate. Suc at 1%, when present in the external solution at 250 μM external nitrate, reduced the flow of root exudate to such an extent that sufficient volumes could not be collected for analysis.

Because the presence of nitrate itself, as well as that of the exogenous effectors, was capable of altering the flow rate of sap during a 1-h collection period, the nitrate flow rate was also determined. Increasing the external nitrate from 250 μM to 5 mM greatly increased the volume of exudate collected, which is reflected in the determination of the nitrate flow rate (Fig. 2B). The presence of Asn and Gln reduced the flow of sap, and the volume collected at 5 mM external nitrate varied from 0.25 mL h^{-1} in amide-treated plants to 1.25 mL

h^{-1} in untreated controls. In amide-treated plants the flow rate was highest immediately after excision of the shoot but declined to less than 25% of the initial rate by the end of the 1-h collection period. This contributed to the extreme differences in the nitrate flow rate in xylem sap during the 1-h collection period between amide-treated plants and untreated controls. However, the concentration of nitrate in the xylem sap collected during the initial 10 min after excision was comparable in the presence and absence of amide.

Effect of Amides and Suc on the Induction of NRA and NiRA

The level of induction of shoot NADH-NRA and root NADH- and NADPH-NRA in the absence of exogenous effectors was approximately the same at 250 μM and 5 mM external nitrate (Fig. 3). This indicates that increasing the concentration of external nitrate does not increase the induced levels of NRA 8 h after exposure to nitrate. At both external nitrate concentrations the induction of shoot and root NRA was significantly reduced by both Asn and Gln. The effect was more pronounced in roots than in shoots. Suc significantly increased the induction of root NRA at both concentrations of external nitrate. The induction of shoot NRA, however, was significantly reduced in the presence of Suc at the lower external nitrate concentration.

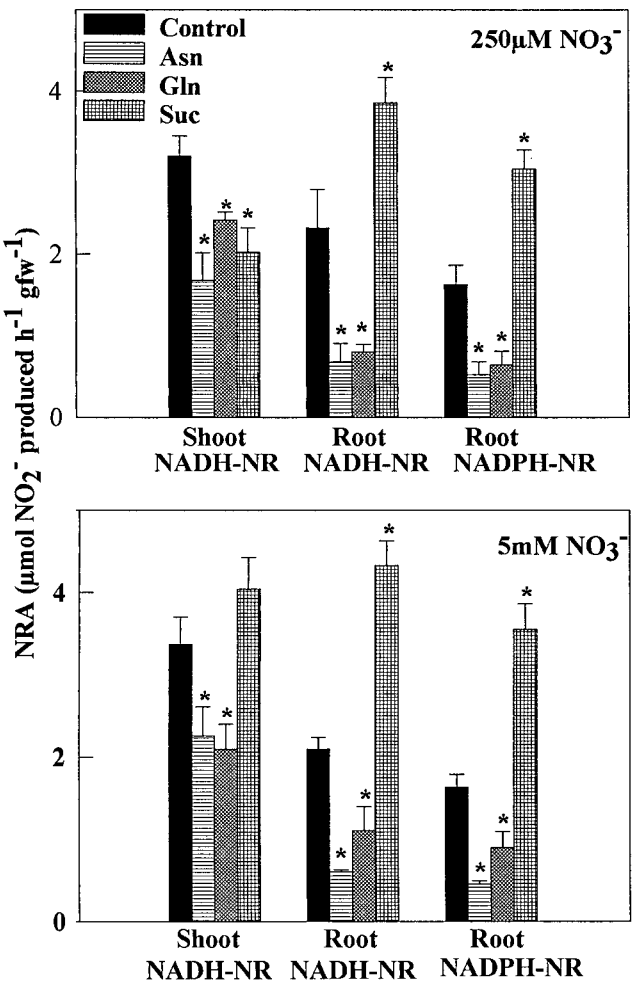

Figure 3. The induction of activities of shoot NADH-NR and root NADH- and NADPH-NR of 6-d-old maize seedlings induced with 250 μM or 5 mM KNO_3 in the presence of 1 mM Asn, 1 mM Gln, or 1% Suc. Seedlings were harvested 8 h after induction. Values represent means ± SD of three separate experiments. gfw, Grams fresh weight; *, significant difference ($P < 0.05$) between amide-/Suc-treated plants and untreated controls.

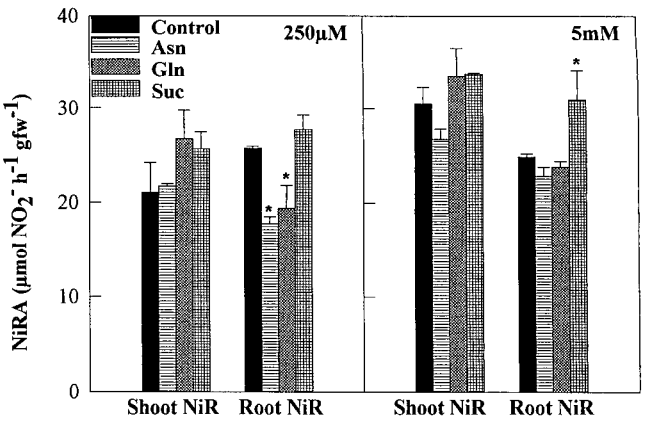

Figure 4. Shoot and root NiRAs in 6-d-old maize seedlings induced with 250 μM or 5 mM KNO_3 in the presence of 1 mM Asn, 1 mM Gln, or 1% Suc. Seedlings were harvested 8 h after induction. Values represent means ± SD of three separate experiments. gfw, Grams fresh weight; *, significant difference ($P < 0.05$) between amide-/Suc-treated plants and untreated controls.

After 8 h of exposure to nitrate, the induction of shoot NiRA was higher at 5 mM than at 250 μM external nitrate (Fig. 4). The level of induction of root NiRA, however, was approximately the same at both external nitrate concentrations. Both Asn and Gln produced a significant reduction in the induction of root NiRA at 250 μM external nitrate. This effect, however, was not observed at 5 mM external nitrate. Suc significantly increased the induction of root NiRA at 5 mM external nitrate.

Effect of Combined Application of Amides and Suc on the Expression of NR and NiR

To determine whether the positive regulatory effect of Suc on nitrate reduction occurs as a result of lower Asn levels in tissue mediated through the negative regulation of Asn synthetase, the expression of NR and NiR mRNA was determined in the presence of Suc, either alone or in combination with amide. Only roots were used for this experiment, because they are more sensitive to these exogenous effectors than shoots (Figs. 3 and 4). Five-day-old maize seedlings were exposed to 1% Suc alone or together with 1 mM Asn or Gln prior to induction with 5 mM nitrate. Transcript levels of NR and NiR were determined 20 h after induction, because the activity of both enzymes in the presence of amides was significantly reduced by this time (Fig. 5A).

In the presence of Suc alone there was an increase in transcript levels of both root NR and NiR, whereas in the presence of either Asn or Gln activities and transcript levels of both enzymes were reduced (Fig. 5, B and C).

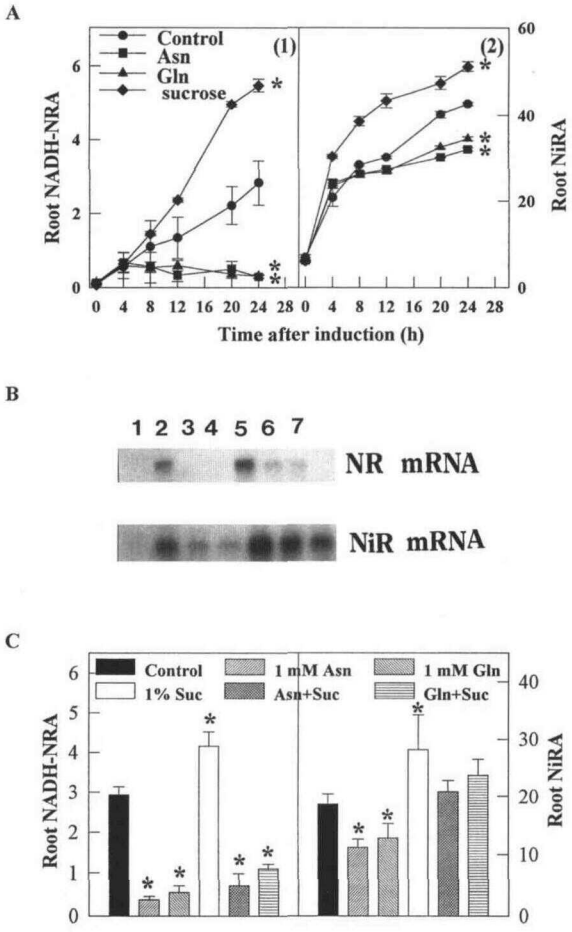

Figure 5. A, Time course for the induction of NADH-NRA (1) and NiRA (2) in roots of 5-d-old maize seedlings in the presence or absence of 1 mM Asn, 1 mM Gln, or 1% Suc. The nitrate concentration was 5 mM. The effectors were added 2 h before nitrate, and tissue was collected at 4-h intervals until 24 h after induction. The seedlings were continuously illuminated. B, Induction of NR and NiR mRNA in roots of 5-d-old maize seedlings. Lane 1, In the absence of nitrate; lane 2, in the presence of 5 mM KNO_3; lane 3, in the presence of 5 mM KNO_3 and 1 mM Asn; lane 4, in the presence of 5 mM KNO_3 and 1 mM Gln; lane 5, in the presence of 5 mM KNO_3 and 1% Suc; lane 6, in the presence of combinations of nitrate, Asn, and Suc at the same concentrations as above; and lane 7, in the presence of combinations of nitrate, Gln, and Suc. The northern blots are representative of two separate experiments. Uniform loading of samples was ensured by ethidium bromide staining of the gel. C, The induction of NADH-NRA and NiRA in seedling roots as influenced by the same treatments as in B. For B and C, seedlings were harvested 20 h after induction with 5 mM KNO_3. *, Significant difference between control and treatments.

When either amide was applied alone NR and NiR transcripts were reduced to similar extents. The addition of Suc together with either amide increased NiRA and transcript above the absolutely inhibited levels observed in the presence of amide alone, and both recovered back to control levels. However, in the case of NR there were only slight increases in activity and transcript above the absolutely inhibited levels, and this was not significant.

Effect of Exogenous Suc and Amides on Endogenous Amide Pools

The addition of 1 mM Asn or Gln increased the endogenous levels of both amides in shoots and roots of maize seedlings, indicating that the amide was absorbed by roots from the external solution and translocated from root to shoot (Table I). Also, there was interconversion between the amides within the seedling tissue after absorption, since the application of either amide led to an increase of both amides. Exogenous Suc reduced Asn concentration in the roots 10 h after its addition, but this reduction was not significant.

DISCUSSION

The expression of nitrate uptake permease(s), NR, and NiR, the first three enzymes in the nitrate assimilatory pathway, is known to be regulated by end products of the nitrate assimilatory pathway, including Gln and Suc (Cheng et al., 1992; Padgett and Leonard, 1993; Vincentz et al., 1993; Li et al., 1995; Sivasankar and Oaks, 1996). Any regulatory factor that affects the nitrate uptake system is bound to affect the expression of NR and NiR as well, since the presence of nitrate within the tissue is a critical determinant of the expression of both enzymes. In the present study we attempted to determine whether the inhibition of NR and NiR expression in the presence of Gln or Asn and their enhancement in the presence of Suc is due to a direct effect on the expression of these genes or due to the regulation of the nitrate uptake system leading to altered tissue nitrate concentrations.

The induction of shoot and root NRA in maize and that of the inducible high-affinity nitrate transport system in barley are both known to approach maximum levels by 8 h after the first exposure to nitrate (Fig. 5A; Henriksen and Spanswick, 1993; Li and Oaks, 1993). Therefore, this time in the linear phase of the induction process was chosen for our experiments. After 8 h of exposure to nitrate in the absence of exogenous effectors, the level of induction of shoot NADH-dependent NRA and root NADH- and NADPH-dependent NRA in 6-d-old maize seedlings was similar at 250 μM and 5 mM external nitrate (Fig. 3). This was in spite of the fact that shoot and root nitrate concentrations increased more that 2-fold in these control plant tissues when external nitrate was increased from 250 μM to 5 mM (Fig. 1). Thus, a nitrate concentration as low as 250 μM was sufficient to produce maximum induction of NRA.

Asn and Gln at concentrations approximating physiological ones produced an 80% inhibition of the induction of NRA in maize root tissue (Fig. 3). However, root tissues were more sensitive to the inhibitory effect of amides than were shoot tissues, in spite of the fact that the amide concentrations in shoot tissues were also elevated by exogenous amide additions, indicating an efficient transfer from the root to the shoot (Table I). Three different possibilities could contribute to the differential effect of amides on the induction of NRA in shoots and roots: (a) differences in the sensitivity of the shoot and root isozymes to amides, (b) differential compartmentation of exogenously fed amides

Table I. *Effect of exogenous Asn, Gln, or Suc on tissue amide concentrations of 6-d-old maize seedlings*

The values represent means ± SD of three separate experiments.

Treatment	Shoot		Root	
	Asn	Gln	Asn	Gln
	μmol g⁻¹ fresh wt			
250 μM KNO_3				
NO_3^- alone	2.51 ± 0.0	0.33 ± 0.0	1.34 ± 0.4	0.41 ± 0.0
NO_3^- + 1mM Asn	5.65 ± 0.7[a]	0.77 ± 0.1[a]	5.19 ± 0.9[a]	1.15 ± 0.1[a]
NO_3^- + 1mM Gln	4.06 ± 0.9[a]	0.64 ± 0.1[a]	2.41 ± 0.9	1.71 ± 0.2[a]
NO_3^- + 1% Suc	2.68 ± 0.7	0.43 ± 0.0	0.99 ± 0.1	0.46 ± 0.1
5 mM KNO_3				
NO_3^- alone	2.86 ± 1.2	0.38 ± 0.0	1.09 ± 0.2	0.33 ± 0.1
NO_3^- + 1mM Asn	5.54 ± 1.0[a]	1.08 ± 0.2[a]	3.11 ± 0.5[a]	1.67 ± 0.3[a]
NO_3^- + 1mM Gln	3.62 ± 1.7	0.67 ± 0.2	2.02 ± 0.3[a]	1.19 ± 0.2[a]
NO_3^- + 1% Suc	2.35 ± 1.1	0.27 ± 0.1	0.92 ± 0.0	0.59 ± 0.1

[a] Significant difference ($P < 0.05$) between amide-/Suc-treated plants and untreated controls.

in shoot and root tissues, and (c) differences in the turnover rates of the shoot and the root enzymes. There is evidence to support the third possibility. If the differential response to amides is a consequence of differences in the turnover rates of the two enzymes, the effect of amides on transcription should be the same. The results of Li et al. (1995) indicate that the addition of Gln to maize seedlings resulted in the differential inhibition of NR protein and NRA in shoots and roots but similar inhibition of transcript in the two organs. Furthermore, the induction and turnover of the NR protein in response to nitrate has been shown to be different in shoots and roots (Li and Oaks, 1993).

The induction of NiRA was also inhibited in the presence of amides, but the effect was less pronounced than in the case of NR. However, NiR mRNA was reduced to the same extent as NR mRNA (Fig. 5B). Therefore, although the regulation of transcription of NR and NiR is the same in the presence of amides, the effect on enzyme activity is different. This is possibly due to the slower turnover rate of the NiR protein relative to the NR protein.

The accumulation of nitrate in seedling tissues was significantly reduced in the presence of Asn at 250 μM external nitrate (Fig. 1). Thus, at 250 μM external nitrate, Asn inhibits both nitrate uptake and reduction, whereas at 5 mM external nitrate, the inhibition is confined to nitrate reduction. The inhibitory effect of Asn on NRA is direct and not the result of an inhibition of uptake as established by the following observations: The level of induction of NRA was similar in untreated control plants at both concentrations of external nitrate, in spite of the higher concentrations of tissue nitrate at 5 mM external nitrate. Although the concentration of tissue nitrate in plants treated with Asn in the presence of 5 mM nitrate was higher than that in control plants exposed to 250 μM nitrate, the induction of NRA remained inhibited.

Gln inhibited the expression of NR and NiR at both concentrations of external nitrate but tissue nitrate concentration remained unaffected. Thus the inhibitory effect of Gln is confined to the nitrate reduction system. Contrary to our results, Lee et al. (1992), using 10 mM exogenous Gln, found an inhibition of nitrate uptake in maize seedlings. The most likely explanation is that the high concentration of applied Gln resulted in a high concentration of tissue Asn due to the conversion of Gln to Asn within the seedling. It is interesting to note that treatments such as phosphate or sulfate deprivation, which result in increased tissue Asn and Gln concentrations, also decrease nitrate uptake (Karmoker et al., 1991; Rufty et al., 1993).

Suc enhanced the expression of both NR and NiR but did not significantly affect nitrate uptake. It has been shown that an inverse correlation exists between Suc and Asn pool sizes in plant tissues, since the addition of Suc to maize embryos decreases Asn level (Oaks and Beevers, 1964), whereas Suc starvation of sycamore cell cultures increases it (Genix et al., 1994). However, after 10 h of exposure to Suc, the Asn concentration in the roots was not significantly reduced in maize seedlings, but the induction of root NRA and NiRA was significantly enhanced (Figs. 3 and 4). This indicates that the up-regulation of NR and NiR expression by Suc is independent of its effect on tissue Asn concentration. This observation is strengthened by the fact that NR and NiR transcript levels in the presence of Suc and Asn together were higher than those in the presence of Asn alone (Fig. 5B). When Suc and Asn were applied together the NiR transcript level was comparable to that of the control level, whereas the NR transcript level showed only a slight increase above the inhibited level observed in the presence of Asn alone.

In conclusion, we have shown that, whereas Asn inhibits both the uptake and reduction of nitrate, Gln inhibits only its reduction. The inhibition of NR and NiR expression by Asn and Gln is the result of some mechanism that must directly affect the expression of these genes and is not an indirect effect occurring through a reduction of tissue nitrate levels resulting from an inhibition of nitrate uptake. Although amides inhibit the induction of NiRA to a lesser extent than the induction of NRA, they reduce transcription of NR and NiR to the same extent. On the other hand, Suc enhances the expression of both NR and NiR. The positive regulation of nitrate reduction by Suc does not occur because of a reduction in tissue Asn levels.

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