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Nitrate Acts as a Signal to Induce Organic Acid Metabolism and Repress Starch Metabolism in Tobacco

Wolf-Rüdiger Scheible,[a] Agustín González-Fontes,[a,1] Marianne Lauerer,[b] Bernd Müller-Röber,[c] Michel Caboche,[d] and Mark Stitt[a,2]

[a] Botanisches Institut der Universität Heidelberg, Im Neuenheimer Feld 360, 69120 Heidelberg, Germany
[b] Lehrstuhl für Pflanzenökologie, Universität Bayreuth, Universitätsstrasse 30, 95440 Bayreuth, Germany
[c] Max-Planck-Institut für Molekulare Pflanzenphysiologie, Karl-Liebknecht-Strasse 25, 14476 Golm, Germany
[d] Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, Centre de Versailles, Route de Saint-Cyr, 78026 Versailles Cedex, France

Nia30(145) transformants with very low nitrate reductase activity provide an in vivo screen to identify processes that are regulated by nitrate. Nia30(145) resembles nitrate-limited wild-type plants with respect to growth rate and protein and amino acid content but accumulates large amounts of nitrate when it is grown on high nitrate. The transcripts for nitrate reductase (NR), nitrite reductase, cytosolic glutamine synthetase, and glutamate synthase increased; NR and nitrite reductase activity increased in leaves and roots; and glutamine synthetase activity increased in roots. The transcripts for phospho*enol*pyruvate carboxylase, cytosolic pyruvate kinase, citrate synthase, and NADP–isocitrate dehydrogenase increased; phospho*enol*pyruvate carboxylase activity increased; and malate, citrate, isocitrate, and α-oxoglutarate accumulated in leaves and roots. There was a decrease of the ADP–glucose pyrophosphorylase transcript and activity, and starch decreased in the leaves and roots. After adding 12 mM nitrate to nitrate-limited Nia30(145), the transcripts for NR and phospho*enol*pyruvate carboxylase increased, and the transcripts for ADP–glucose pyrophosphorylase decreased within 2 and 4 hr, respectively. Starch was remobilized at almost the same rate as in wild-type plants, even though growth was not stimulated in Nia30(145). It is proposed that nitrate acts as a signal to initiate coordinated changes in carbon and nitrogen metabolism.

INTRODUCTION

Nitrate is the major source of nitrogen in most plants (Marschner, 1995). The addition of nitrate leads to increased rates of nitrate uptake (Jackson et al., 1973; Siddiqi et al., 1990; Lainé et al., 1995) and increased activities of nitrate reductase (NR) (Shaner and Boyer, 1976; Galangau et al., 1988; Gowri et al., 1992; Lin et al., 1994), nitrite reductase (NiR) (Wray, 1993), glutamine synthetase (GS), and glutamate synthase (GOGAT) (Hecht et al., 1988; Sakakibara et al., 1991; Hayakawa et al., 1992). There are major alterations in carbon metabolism, including a decrease of starch (Hofstra et al., 1985; Waring et al., 1985; Fichtner and Schulze, 1992; Stitt and Schulze, 1994) and the synthesis of organic acids (Foyer and Ferrario, 1994), to provide carbon skeletons for amino acid synthesis and to act as counteranions and prevent alkalinization (Figure 1). Whole-plant allocation and development are also modified by nitrate. Application of nitrate to a restricted part of the root leads to local stimulation of lateral root growth (Drew and Saker, 1975; Granato and Raper, 1989). A general application of high nitrate leads to a preferential stimulation of shoot growth and inhibition of root growth (Lambers et al., 1990; Scheible et al., 1997a) and delays flowering and senescence (Bernier et al., 1993). These far-reaching changes imply that nitrogen metabolism generates signals that regulate metabolism, allocation, and development (Redinbaugh and Campbell, 1991; Crawford, 1994, 1995; Hoff et al., 1994). To define these internal signaling networks, it will be necessary to identify which processes are regulated by nitrate itself and which are influenced indirectly as a result of the metabolism of nitrate to ammonium and amino acids or, even more indirectly, as a result of increased growth.

In fungi (Crawford and Arst, 1993; Marzluf, 1993) and Chlamydomonas (Fernandez and Cardenas, 1989), nitrate induces the expression of the genes that are involved in its own uptake and reduction, including the nitrate transporter NR (*nia*) and NiR (*nii*). These genes are repressed by downstream metabolites formed during nitrate assimilation, such

[1] Current address: Departamento de Biología Vegetal y Ecología, Universidad de Còrdoba, Avenida San Alberto Magno, 14004 Còrdoba, Spain.
[2] To whom correspondence should be addressed. E-mail mstitt@botanik1.bot.uni-heidelberg.de; fax 49-6221-545859.

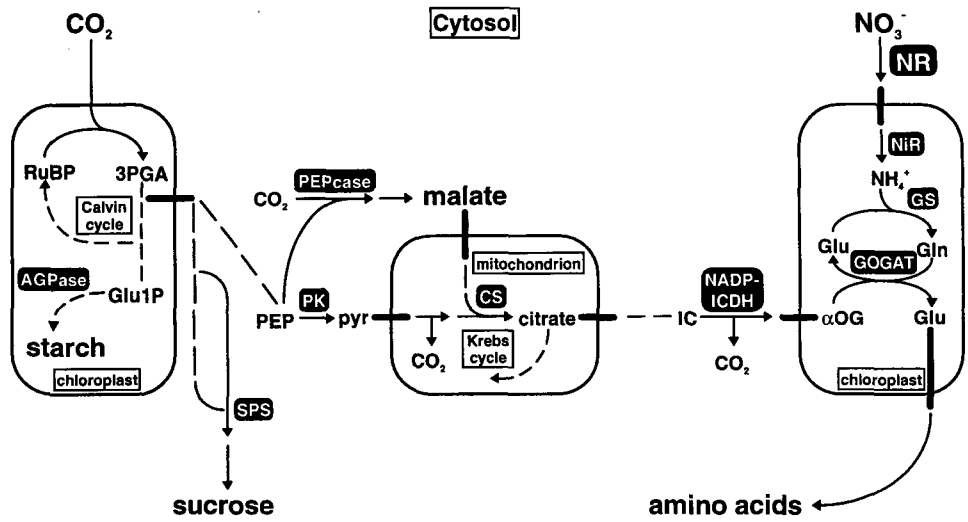

Figure 1. Pathways of Primary Nitrogen and Carbon Metabolism in an Autotrophic Plant Cell.

The scheme shows the main enzymes, including NR, NiR, GS, GOGAT, NADP-dependent isocitrate dehydrogenase (NADP-ICDH), citrate synthase (CS), pyruvate kinase (PK), PEPcase, SPS, and AGPase, and key metabolites including glutamine (Gln), glutamate (Glu), α-oxoglutarate (αOG), isocitrate (IC), pyruvate (pyr), PEP, glycerate-3-phosphate (3PGA), glucose 1-phosphate (Glu1P), and ribulose-1,5-bisphosphate (RuBP). Enzymatic reactions are shown as thin black lines, and transport processes are shown as bold black lines.

as glutamine. A similar picture is emerging in higher plants. The addition of nitrate leads to a rapid increase in the transcripts for the high-affinity nitrate transporter (Trueman et al., 1996; Quesada et al., 1997), *nia* (Pouteau et al., 1989; Cheng et al., 1991; Gowri et al., 1992; Lin et al., 1994), and *nii* (Rastogi, 1993; Wray, 1993). This response involves primary sensing, because the transcripts increase when nitrate is added to NR-deficient genotypes (Pouteau et al., 1989; Vaucheret et al., 1990; Kronenberger et al., 1993; Quesada et al., 1997), and the increase is insensitive to protein synthesis inhibitors for *nia* (Gowri et al., 1992). The addition of ammonium or glutamine leads to a decrease in transcripts for the high-affinity nitrate transporter (Quesada et al., 1997), *nia* (Hoff et al., 1994), and *nii* (Kronenberger et al., 1993). This explains why the nitrate-induced increase of these transcripts is rapidly reversed in wild-type plants but not in *nia* mutants (Vaucheret et al., 1990; Quesada et al., 1997).

It is not known whether nitrate regulates other events in carbon and nitrogen metabolism in plants. The addition of nitrate leads to a rapid, cycloheximide-insensitive increase in *gln2* (encoding plastid glutamine synthetase [GS2]) and *glu* transcripts (ferredoxin-dependent glutamate synthase [Fd-GOGAT]) in maize roots but not in leaves (Redinbaugh and Campbell, 1993). It leads to an increase in the *ppc* transcript (encoding phospho*enol*pyruvate carboxylase [PEPcase]) in maize leaves (Sugiharto and Sugiyama, 1992) and the *icdh1* transcript (encoding cytosolic NADP–isocitrate dehydrogenase [NADP-ICDH]) in potato leaves (Fieuw et al., 1995). However, these changes could be due to metabolites formed during nitrate assimilation. The addition of ammonium or glutamine led to an even larger increase of the *ppc* transcript in maize leaves (Sugiharto et al., 1992), indicating that *ppc* is not induced by nitrate itself.

Carbon and nitrogen metabolism are also regulated by protein phosphorylation (Champigny and Foyer, 1992; Kaiser and Huber, 1994; Huber and Huber, 1996). The addition of nitrate leads to activation of PEPcase (Van Quy and Champigny, 1992; Duff and Chollet, 1995) and inactivation of sucrose phosphate synthase (SPS) (Champigny et al., 1992; Huber and Huber, 1996). However, these changes may be due to metabolites formed during nitrate assimilation (Foyer et al., 1996). SPS is inactivated when ammonium is supplied to detached leaves (Champigny et al., 1992), and glutamine promotes the light activation of PEPcase kinase and subsequent phosphorylation and activation of PEPcase in tobacco leaves (Li et al., 1996).

In the experiments described in this study, tobacco transformants with very low NR activity were used to investigate whether carbon–nitrogen interactions are regulated by signals deriving from nitrate per se. In these plants, the nitrate supply can be varied independent of the rate of nitrate assimilation, the levels of downstream metabolites, such as glutamine and ammonium, and growth. We investigated whether nitrate leads to widespread changes in the expression of key genes in the pathways of nitrogen and carbon metabolism and whether these result in significant changes in the activities of the encoded enzymes.

RESULTS

Plant Growth and Nitrogen Content

Wild-type tobacco has two loci for NR at *nia1* and *nia2*. The double mutant Nia30 (*nia1*$^-$/*nia1*$^-$ *nia2*$^-$/*nia2*$^-$; Müller and Mendel, 1989) was transformed by Vaucheret et al. (1990) with a 12-kb construct containing a 5-kb upstream promoter sequence, the structural gene, and a 2-kb downstream section from *nia2* to produce the transformant Nia30(145). The reintroduced *nia* gene is only weakly expressed (Vaucheret et al., 1990; Vaucheret and Caboche, 1995; Scheible et al., 1997a). Typical NR activities for the wild-type plants and Nia30(145) were 45 ± 5 and 0.8 ± 0.5 μmol hr^{-1} g fresh weight of tissue^{-1} in 12 mM nitrate and 4 ± 1 and 0.5 ± 0.3 μmol hr^{-1} g fresh weight of tissue^{-1} in 0.2 mM nitrate, respectively (data not shown). The defective copies of *nia1* and *nia2* are still transcribed and translated to produce a nonfunctional protein (Müller and Mendel, 1989; Vaucheret et al., 1990; Scheible et al., 1997b).

The growth rate (Figure 2A), the protein content (Figure 2B), amino acids (Figure 2C), glutamine (Figure 2E), chlorophyll (data not shown), Rubisco activity (data not shown), and the rate of photosynthesis (data not shown) of Nia30(145) resemble those of nitrogen-deficient wild-type plants (see also Scheible et al., 1997a). Whereas these parameters increased in response to nitrate fertilization in the wild type, they did not change significantly in Nia30(145) when the nitrate supply was increased from 0.2 to 12 mM. Rather, large amounts of nitrate accumulated in the transformant (Figure 2D). Similar results were obtained for another transformant Nia30(461) and a double mutant Nia34 (Lauerer, 1996; Scheible et al., 1997a).

The accumulation of nitrate affects the osmotic potential. To minimize such effects, when nitrate was reduced from 12 to 0.2 mM, it was replaced by a mixture of magnesium sulfate and potassium chloride to maintain the same individual cation concentration and the same overall anion concentration in the nutrient medium. The total concentration of inorganic ions in the leaves was 0.40 mmol g fresh weight^{-1} in wild-type plants grown on 12 mM nitrate, and 0.82, 0.71, and 0.81 mmol g fresh weight^{-1} in wild-type plants grown in 0.2 mM nitrate and Nia30(145) grown on 12 and 0.2 mM nitrate, respectively. The total concentration of ions rises under conditions in which growth is decreased (compare with Figure 2A), but it is not specifically increased when nitrate accumulates in Nia30(145).

Comparison of wild-type plants grown on low nitrate with low NR activity transformants grown on high nitrate should therefore provide an in vivo screen for processes that are regulated by nitrate signaling. The nitrate dependence of such changes can be checked by investigating whether they are reduced or abolished when Nia30(145) is grown on low nitrate or in the absence of nitrate and whether they appear rapidly after supplying nitrate to nitrate-deficient plants (see below).

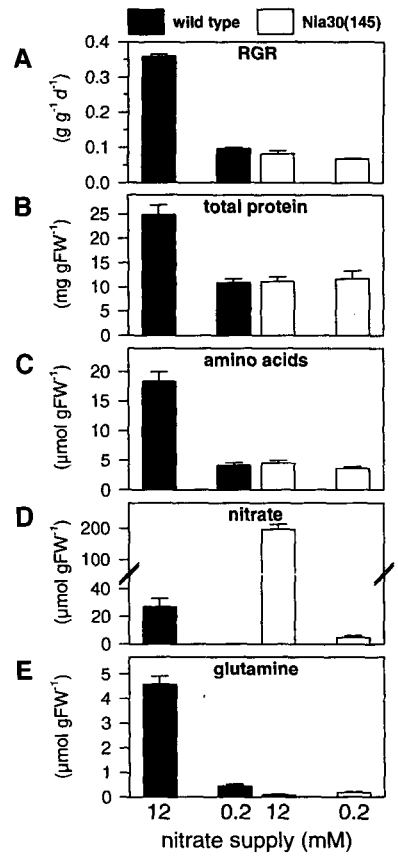

Figure 2. Growth and Composition of Wild-Type Plants and Severely NR-Deficient Transformants Grown on High and Low Nitrate.

Wild-type plants and Nia30(145) were grown as described in Methods on nutrient medium containing 12 or 0.2 mM nitrate and harvested at the rosette stage, when they were in the exponential growth stage. To obtain plants at this stage in their development, the samples were taken after ~32 days (wild-type plants on 12 mM nitrate), 62 days (wild-type plants on 0.2 mM nitrate and Nia30(145) on 12 mM nitrate), or 85 to 90 days (Nia30(145) on 0.2 mM nitrate) after germination. Protein, amino acids, and nitrate were measured in samples taken after 4 hr of illumination in the first fully expanded leaf and are related to the fresh weight (FW) of the tissue. The relative growth rate is given as the relative daily increase in dry weight (g g^{-1} d^{-1}, gram dry weight · gram dry weight $^{-1}$ · day $^{-1}$). The results are the mean ± SE of three experiments, each with four separate plants.

(A) Relative growth rate (RGR).

(B) Protein content.

(C) Overall content of amino acids.

(D) Nitrate content.

(E) Glutamine content.

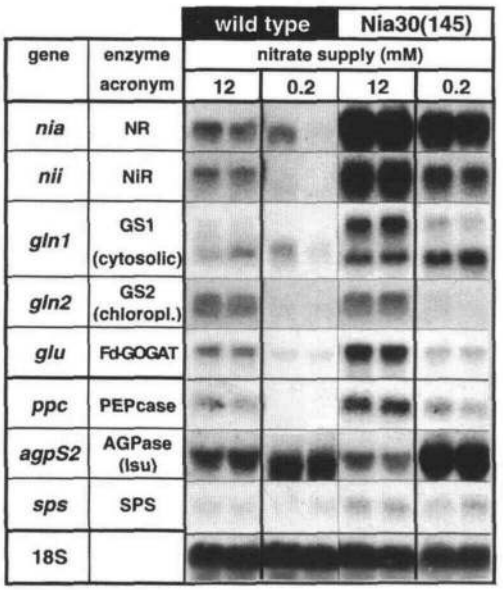

Figure 3. Nitrate Accumulation in Severely NR-Deficient Transformants Induces Genes Encoding Enzymes for Nitrate and Ammonium Assimilation, Induces PEPcase, and Represses AGPase in Source Leaves.

Transcripts for *nia*, *nii*, *gln1*, *gln2*, *glu*, *ppc*, *agpS2*, *sps*, and 18S as a control are shown for RNA preparations from the youngest fully expanded leaves of two separate groups of plants. The plants were grown as described in the legend to Figure 2, and samples were taken after 4 hr of illumination. The levels of nitrate and glutamine are shown in Figures 2D and 2E. chloropl., chloroplast.

Transcripts for Key Enzymes in Nitrate Assimilation, Organic Acid Metabolism, and Carbohydrate Metabolism

The transcripts for *nia* and *nii* were low in nitrate-deficient wild-type plants and high in Nia30(145) grown on 12 mM nitrate (Figure 3). Both transcripts decreased when Nia30(145) was grown on 0.2 mM nitrate, although not as far as in nitrate-deficient wild-type plants. Nia30(145) on 0.2 mM nitrate contained more nitrate than did wild-type plants on 0.2 mM nitrate (Figure 2D). The transcripts almost disappeared when Nia30(145) was grown on ammonium (Figure 4A) to prevent accumulation of nitrate (Figure 4B).

Wild-type plants growing on low nitrate contained low transcripts for cytosolic glutamine synthetase (GS1) (*gln1*) and Fd-GOGAT (*glu*) (Figure 3). Both transcripts increased in Nia30(145) grown on 12 mM nitrate, and this increase was reversed on 0.2 mM nitrate. The increase of GS1 was partly due to the appearance of a new slower running band. The GS2 transcript (*gln2*) was only slightly increased in Nia30(145) compared with a nitrate-deficient wild type (Figure 3).

Nitrate-deficient wild-type plants contained low levels of transcripts for PEPcase (*ppc*) (Figure 3). Transcripts for PEPcase increased dramatically when Nia30(145) was grown on 12 mM nitrate, and this increase was reversed on 0.2 mM nitrate (Figure 3). Nia30(145) grown on 12 mM nitrate also contained high levels of transcripts for NADP-ICDH (*icdh1*), mitochondrial citrate synthase (cs_m), and cytosolic pyruvate kinase (pk_c) (Figure 5A). The sensitivity of genes involved in nitrate assimilation and organic acid metabolism to nitrate induction was further investigated by using F23×Nia30 (Figure 5). This mutant had about half of the wild-type NR activity (Scheible et al., 1997a) and a growth rate similar to that of the wild type, and it contained slightly elevated pools of nitrate and slightly less glutamine (Figures 5B and 5C). The increase in *ppc*, *icdh1*, cs_m, and pk_c transcripts in F23×Nia30 was as great as that of *nia* and *nii* (Figure 5A).

The transcript for the large subunit of ADP glucose pyrophosphorylase (*agpS2*) was high in nitrate-deficient wild-type plants and decreased in Nia30(145) grown on 12 mM nitrate; this decrease was reversed when the nitrate supply was decreased (Figures 3 and 4A). This repression of *agpS2* does not represent a general repression of carbohydrate synthesis. The *sps* transcript did not decrease when nitrate accumulated in Nia30(145) (Figure 3).

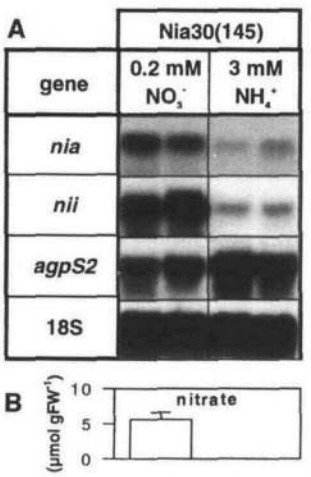

Figure 4. Growth of Nia30(145) on Ammonium Leads to a Large Decrease in the Transcripts for NR and NiR and a Further Increase of the Transcript for AGPase.

Nia30(145) was grown on 0.2 mM nitrate or 3 mM ammonium as the sole source of nitrogen.The transcripts are shown for RNA preparations from the youngest fully expanded leaves of two separate groups of plants. The samples were taken at midday. Nitrate levels are related to the fresh weight (FW) and are given as the mean ± SE ($n = 4$).

(A) *nia*, *nii*, *agpS2*, and 18S transcripts.

(B) Nitrate content.

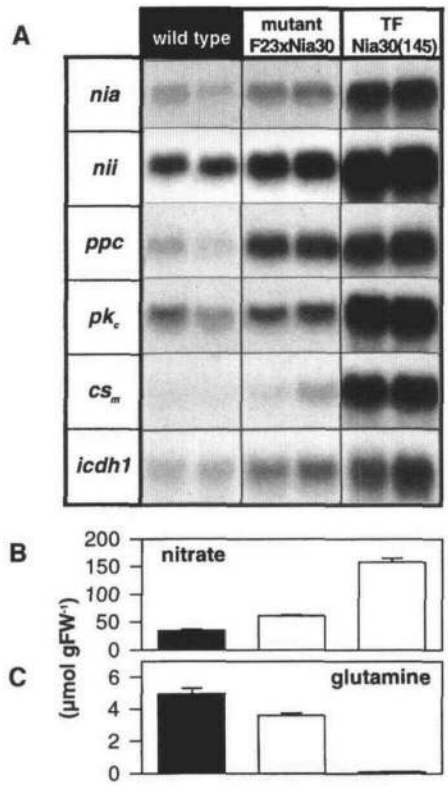

Figure 5. Genes Needed for Nitrate Assimilation and Organic Acid Metabolism Are Also Induced in Mutants with a Moderate Decrease of NR Activity.

Transcripts were measured in RNA preparations from the youngest fully expanded leaves of two separate groups of plants, grown on 12 mM nitrate, and harvested after 4 hr of illumination. NR activity in the mutant F23×Nia30 with one functional *nia* gene was 42 ± 2% of that in the wild-type plants (data not shown). The levels of nitrate and glutamine are related to the fresh weight (FW) and are the mean ± SE ($n = 4$).

(A) Transcripts for NR, NiR, PEPcase, pk_c, cs_m, and cytosolic NADP-ICDH (*icdh1*).

(B) Nitrate content.

(C) Glutamine content.

Roots of Nia30(145) grown on 12 mM nitrate had twofold more nitrate than did roots of wild-type plants grown on 12 mM nitrate (Table 1). Their glutamine levels resembled those in the roots of wild-type plants grown on 0.2 mM nitrate (Table 1). There were no major differences between the total ion concentrations in the roots of wild-type plants grown on 12 and 0.2 mM nitrate and of Nia30(145) grown on 12 and 0.2 mM nitrate (0.17, 0.18, 0.19, and 0.2 mmol g fresh weight^{-1}, respectively). The response of the transcripts in the roots (data not shown) resembled that in the leaves. Roots of nitrate-limited wild-type plants contained low transcript levels for *nia*, *nii*, *gln1*, and *ppc* and high *agpS2* transcript levels. Roots of Nia30(145) grown on 12 mM nitrate contained high levels of transcripts for *nia*, *nii*, *gln1*, and *ppc* and a low level of the *agpS2* transcript. These changes were partly (*nia* and *nii*) or completely (*gln1*, *ppc*, and *agpS2*) reversed when Nia30(145) was grown on 0.2 mM nitrate.

Changes in Enzyme Activities

We next investigated whether these changes in transcript levels are accompanied by increased activities of key enzymes in nitrogen and carbon metabolism. Because the NR protein produced by the point mutated *nia* genes in Nia30(145) is inactive, the effect of nitrate on *nia* expression was monitored by measuring NR protein levels.

Leaves of nitrate-deficient wild-type plants (0.2 mM nitrate) have low amounts of the NR protein (Figure 6A) and low NiR (Figure 6B) and PEPcase (Figure 6D) activities. Leaves of Nia30(145) grown on 12 mM nitrate contain large amounts of the NR protein and have very high NiR and PEPcase activities (Figures 6A, 6B, and 6D). The NR protein is double that in the wild type on a fresh weight basis and fourfold higher on a protein basis (compare Figures 2B and 6A). NiR and PEPcase activities were up to 10-fold higher than in the nitrate-deficient wild-type plants, irrespective of whether the results are expressed on a fresh weight or a protein basis (compare Figures 6B and 6D with Figure 2B). These changes were reversed when Nia30(145) was grown on 0.2 mM nitrate (Figures 6A, 6B, and 6D). Total GS activity did not increase in Nia30(145) compared with nitrogen-deficient wild-type plants (Figure 6C).

Similar measurements were conducted with the roots. The NR protein (data not shown) and NiR and PEPcase activities (Table 1) were low in nitrate-limited wild-type plants, high in Nia30(145) grown on 12 mM nitrate, and low in Nia30(145) grown on 0.2 mM nitrate. GS activity was low in nitrate-limited wild-type plants and increased fourfold in roots when Nia30(145) was grown on 12 mM nitrate, reaching a higher activity than in well-fertilized wild-type plants (Table 1).

Wild-type plants grown on 0.2 mM nitrate have high AGPase (Figure 6E) and low SPS (Figure 6F) activity. In contrast, Nia30(145) grown on 12 mM nitrate has low AGPase (Figure 6E) and high SPS (Figure 6F) activity. The ratios of PEPcase/AGPase activities (Figure 7A) and SPS/AGPase activities (Figure 7B) are 20- and fivefold higher, respectively, in Nia30(145) grown on 12 mM nitrate than in nitrate-deficient wild-type plants and resemble those found in wild-type plants grown on 12 mM nitrate.

PEPcase and SPS are regulated by protein phosphorylation. Phosphorylation activates PEPcase by decreasing the sensitivity to inhibition by malate (Chollet et al., 1996) and inactivates SPS by increasing sensitivity to inhibition by phosphate (Pi) (Huber and Huber, 1996). As previously seen (Duff and Chollet, 1995), PEPcase was sensitive to malate in

leaves of nitrate-deficient wild-type plants (Figure 6G). PEPcase in leaves from Nia30(145) grown on 12 mM nitrate was much less sensitive to malate, and sensitivity was restored when Nia30(145) was grown on 0.2 mM nitrate (Figure 6G). Similar changes of PEPcase activity and malate sensitivity occurred in sink leaves (data not shown). The Pi sensitivity of SPS was not affected (Figure 6H).

Organic Acid Metabolism

Growth of wild-type plants on 0.2 mM nitrate led to a large decrease of α-oxoglutarate, isocitrate, citrate, and malate (Figures 6I to 6L). Compared with nitrate-deficient wild-type plants, Nia30(145) grown on 12 mM nitrate contained eight- to 10-fold more α-oxoglutarate, fourfold more isocitrate, two- to threefold more citrate, and slightly more malate (Figures 6I to 6L). Pyruvate, PEP, and glycerate 3-phosphate (3PGA) (Figures 6M to 6O) decreased in Nia30(145) grown on 12 mM nitrate compared with nitrate-deficient wild-type plants. Accumulation of nitrate in leaves of Nia30(145) therefore leads to a marked increase in the organic acids that are precursors for the GOGAT pathway (α-oxoglutarate and isocitrate), a smaller but significant accumulation of the organic acids that normally accumulate in large amounts as counteranions (malate and citrate) during nitrate assimilation, and a decrease in the glycolytic intermediates (3PGA, PEP, and pyruvate) that act as precursors for organic acid synthesis.

Organic acid metabolism was also altered in the roots (Table 1). When wild-type plants were grown on low nitrate, there was a large decrease in malate and citrate and a smaller decrease in isocitrate and α-oxoglutarate. These organic acids increased by three- to 10-fold in Nia30(145) transformants grown on 12 mM nitrate, and these increases were reversed on 0.2 mM nitrate. Organic acid metabolism was also modified in sink leaves (data not shown). Sink leaves of Nia30(145) grown on 12 mM nitrate also contained fourfold more α-oxoglutarate and twofold more isocitrate than did sink leaves of wild-type plants grown on 0.2 mM nitrate (data not shown).

Starch Metabolism

Source leaves of wild-type plants on 0.2 mM nitrate contained large amounts of starch (Figure 6P) and relatively low levels of sugars (Figures 6Q and 6R) at midday. In contrast, starch was very low in leaves of Nia30(145) grown on 12 mM nitrate (Figure 6P). This decrease was reversed when Nia30(145) was grown on 0.2 mM nitrate. The low starch content of Nia30(145) on 12 mM nitrate was not due to a general depletion of carbohydrates in the leaves. Sucrose was unaltered (Figure 6Q), and reducing sugars were higher (Figure 6R) than in nitrate-deficient wild-type plants. Similar starch changes were found in the roots (Table 1).

Starch is synthesized in leaves during the photoperiod and degraded during the night. Starch levels at the beginning and end of the photoperiod were investigated in a separate experiment (Figure 8). In this experiment, two mutant lines (F22×F23 and F23×Nia30) that had a slightly reduced NR expression were included, and the plants were grown at 12, 1.6, or 0.2 mM nitrate. In wild-type plants, low-nitrate fertilization led to a higher starch content at the start (Figure 8A)

Table 1. Alterations of Enzyme Activities and Metabolites in the Roots of Wild-Type Plants and Nia30(145) Grown on 12 and 0.2 mM Nitrate[a]

Parameter	Wild Type		Nia30(145)	
Nitrate nutrition (mM)	12	0.2	12	0.2
Nitrate[b]	27.9 ± 5.6	0.4 ± 0.2	68.0 ± 7.3	1.8 ± 1.0
Glutamine[b]	1.84 ± 0.14	0.14 ± 0.02	0.24 ± 0.03	0.11 ± 0.01
Protein[c]	5.0 ± 0.4	2.1 ± 0.1	5.5 ± 0.5	2.5 ± 0.2
NiR activity[d]	41.3 ± 5.7	12.3 ± 4.5	110.4 ± 5.1	35.7 ± 12.4
GS activity[d]	36.1 ± 3.6	11.4 ± 1.8	53.6 ± 4.7	12.9 ± 1.1
PEPcase activity[d]	27.2 ± 0.9	5.5 ± 0.4	68.5 ± 4.5	5.9 ± 0.5
α-Oxoglutarate[e]	101 ± 13	67 ± 1	230 ± 20	58 ± 7
Isocitrate[e]	36 ± 2	10 ± 1	130 ± 3	11 ± 1
Citrate[b]	3.3 ± 0.2	0.4 ± 0.1	5.2 ± 0.4	0.6 ± 0.0
Malate[b]	12.9 ± 1.0	1.2 ± 0.0	10.4 ± 0.6	1.0 ± 0.1
Starch[f]	1.8 ± 0.1	7.7 ± 0.4	1.1 ± 0.0	10.6 ± 1.0

[a] Results are given as mean values ± SE (n = 6).
[b] Results in micromoles per gram fresh weight of tissue.
[c] Results in milligrams per gram fresh weight of tissue.
[d] Results in micromoles per hour per gram fresh weight of tissue.
[e] Results in nanomoles per gram fresh weight of tissue.
[f] Results in hexose equivalents per gram fresh weight of tissue.

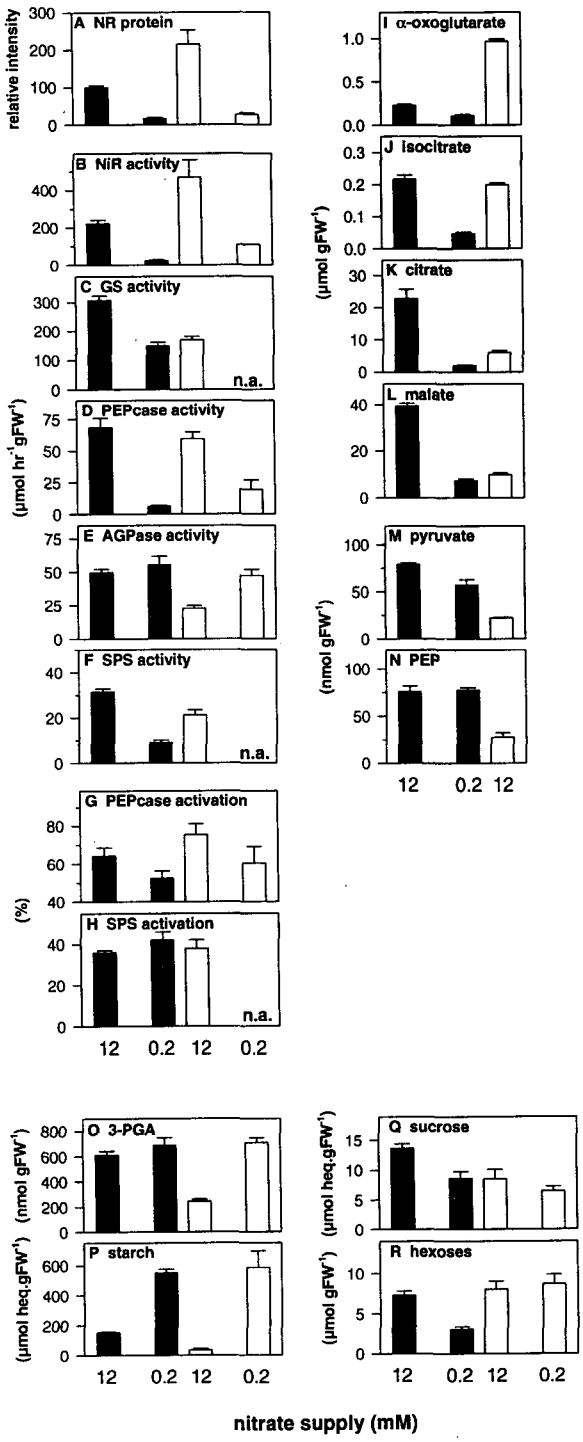

Figure 6. Alterations of Enzyme Activities and of Metabolites in the Source Leaves of Wild-Type Plants and Nia30(145) Transformants Grown on 12 and 0.2 mM Nitrate.

All measurements were performed on samples taken from the youngest fully expanded leaf of the plants after 4 hr of illumination at the same time as samples were taken for measurements of the transcripts shown in Figure 3. For plant age and sampling, see legends to Figures 2 and 3. The results are related to fresh weight (FW) and are the mean ± SE of four to six separate plants. n.a., not analyzed.

(A) NR protein.
(B) NiR activity.
(C) Total GS activity.
(D) PEPcase activity.
(E) AGPase activity.
(F) SPS activity.
(G) Activation of PEPcase.
(H) Activation of SPS.
(I) α-Oxoglutarate content.
(J) Isocitrate content.
(K) Citrate content.
(L) Malate content.
(M) Pyruvate content.
(N) PEP content.
(O) 3PGA content.
(P) Starch content.
(Q) Sucrose content.
(R) Reducing sugars (glucose plus fructose).

and end (Figure 8B) of the day. The rate of synthesis during the 12-hr photoperiod can be estimated from the difference between the starch content at the end of the night and the content at the end of the day (Figure 8C). Low nitrate led to a small stimulation of starch turnover in wild-type plants. Nia30(145) grown on 1.6 or 12 mM nitrate contained very little starch in the leaves at the start (Figure 8A) and end (Figure 8B) of the day and had an extremely low rate of starch synthesis (Figure 8C). Nia30(145) grown on 0.2 mM nitrate resembled a nitrate-limited wild-type plant (Figures 8A to 8C). Figures 8D and 8E compare the starch and nitrate content of the leaves. There is an inverse hyperbolic relationship between leaf nitrate and leaf starch, irrespective of whether leaf nitrate is changing as a result of altered nitrate fertilization or decreased expression of NR. There is an inverse relationship between the leaf nitrate content and the rate of starch synthesis (Figure 8F).

Rapid Changes of Transcripts in Leaves after Supplying Nitrate to Intact Nitrate-Deficient Plants

To investigate the kinetics of these nitrate-induced changes in expression and metabolism, plants were grown on 0.2 mM nitrate for 9 weeks and then transferred to 12 mM nitrate. The nitrate was added 2 hr after the start of the photoperiod, and samples were taken from leaves at various intervals during the remainder of the first photoperiod and after 4 hr of illumination on the subsequent days. The addition of 12 mM nitrate did not stimulate growth of Nia30(145) (Figure 9A; see also Figure 2A). Nitrate accumulated linearly after a short lag of ~1 hr (Figure 9B, inset), reaching levels after 4 days (Figure 9B) similar to those found when Nia30(145)

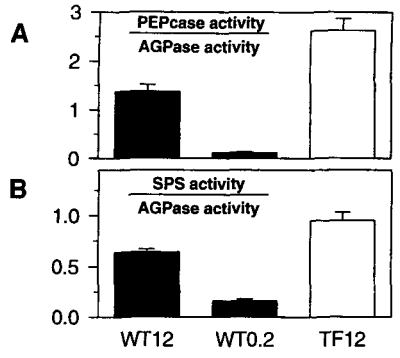

Figure 7. Ratio of AGPase Activity to PEPcase and SPS Activity in Source Leaves.

The ratios of enzyme activities are calculated from the data shown in Figure 6 for wild-type plants grown on 12 mM nitrate (WT12), wild-type plants grown on 0.2 mM nitrate (WT0.2), and Nia30(145) grown on 12 mM nitrate (TF12).
(A) PEPcase/AGPase.
(B) SPS/AGPase.

was grown continuously on 12 mM nitrate (Figure 2D). In wild-type plants, nitrate accumulation was slightly slower on the first day (Figure 9B, inset), much slower on the second day, and reversed from day 3 onward when the plants started to grow faster (Figure 9A).

As previously seen (Pouteau et al., 1989; Vaucheret et al., 1990), the addition of 12 mM nitrate led within 2 hr to a large increase in *nia* transcript in the leaves of wild-type plants and Nia30(145). The *nia* transcript declined after 8 hr in wild-type plants but remained high in Nia30(145) (Figure 10). The *ppc* transcript also increased in both genotypes within 2 hr after the addition of 12 mM nitrate (Figure 10). This increase occurred before the leaf nitrate content had reached 6 μmol g fresh weight^{-1} (Figure 9B), which is negligible compared with the total content of inorganic ions in the leaf (810 μmol g fresh weight^{-1}; data not shown). In wild-type plants, the *ppc* transcript reached a peak after ~4 hr and then declined slightly (Figure 10). A similar decline was found toward the end of the photoperiod in wild-type plants that were watered every day with 12 mM nitrate (data not shown). This decay of the *ppc* transcript was abolished in Nia30(145) (Figure 9B).

The *agpS2* transcript showed a diurnal pattern in wild-type plants and Nia30(145) grown on 0.2 mM nitrate. It rose to a high level after 4 hr of illumination (equivalent to the 2-hr point in Figure 10) and declined slightly toward the end of the photoperiod. The increase after illumination is probably due to sugar induction (Müller-Röber et al., 1990). The transient decrease correlates with a small transient increase of nitrate in the leaf after irrigation with 0.2 mM nitrate (Scheible et al., 1997b). The addition of 12 mM nitrate led to an almost complete disappearance of *agpS2* transcript after 4 to 10 hr. This decrease was found in wild-type plants and in Nia30(145) (Figure 10) and occurred before nitrate rose above 12 μmol g leaf fresh weight^{-1}.

The resulting changes in metabolism are shown in Figure 9. Nia30(145) started to accumulate α-oxoglutarate within 1 day of adding 12 mM nitrate (Figure 9C). Starch remobilization commenced within 1 day in wild-type plants (Figure 9D), before their growth was significantly increased (see Figure 9A). Starch remobilization in Nia30(145) was almost as fast as in wild-type plants, even though growth of Nia30(145) was not altered.

DISCUSSION

Genotypes with Low NR Activity Provide an in Vivo Screen for Nitrate-Regulated Processes

These experiments were performed to investigate whether nitrate acts as a source of signals to regulate carbon and nitrogen metabolism in higher plants. Many earlier investigators have added nitrate to wild-type plants and investigated the resulting changes in metabolism and growth. The interpretation of such experiments is complicated, because it is difficult to distinguish between events that are triggered by nitrate and changes that are produced more indirectly as a result of the assimilation of nitrate and the ensuing changes in cell metabolism and plant growth. Genotypes with low NR activity provide a simpler experimental system because the nitrate content can be varied without causing major changes in the level of organic nitrogen compounds in the plant and the rate of growth. These plants are physiologically nitrogen limited, and they resemble nitrate-deficient wild-type plants with respect to the processes that are regulated by signals derived from organic nitrogenous compounds. They resemble nitrate-replete wild-type plants with respect to the processes that are regulated by signals derived from nitrate itself.

Genotypes with low NR activity are preferable to totally NR-deficient mutants because the latter can only be grown on ammonium or other reduced nitrogen sources, which could themselves act as a source of signals (Hoff et al., 1994), and because wild-type plants and NR-deficient genotypes differ in their ability to balance the rates of nitrate and ammonium assimilation.

The interpretation of our results could be complicated if nitrate accumulation leads to osmotic effects. This cannot be the explanation for the changes in nitrogen, organic acid, and starch metabolism investigated in this article. The overall ion concentration in the mature leaves and roots of Nia30(145) grown on 12 mM nitrate resembled the concentrations in the leaves and roots of Nia30(145) or wild-type plants grown on 0.2 mM nitrate. Furthermore, similar trends in the levels of transcripts were seen in mutants with a relatively small decrease in NR activity and increased nitrate, and the addition of nitrate to nitrate-deficient wild-type plants or Nia30(145) led to changes in *nia*, *ppc*, and *agpS2*

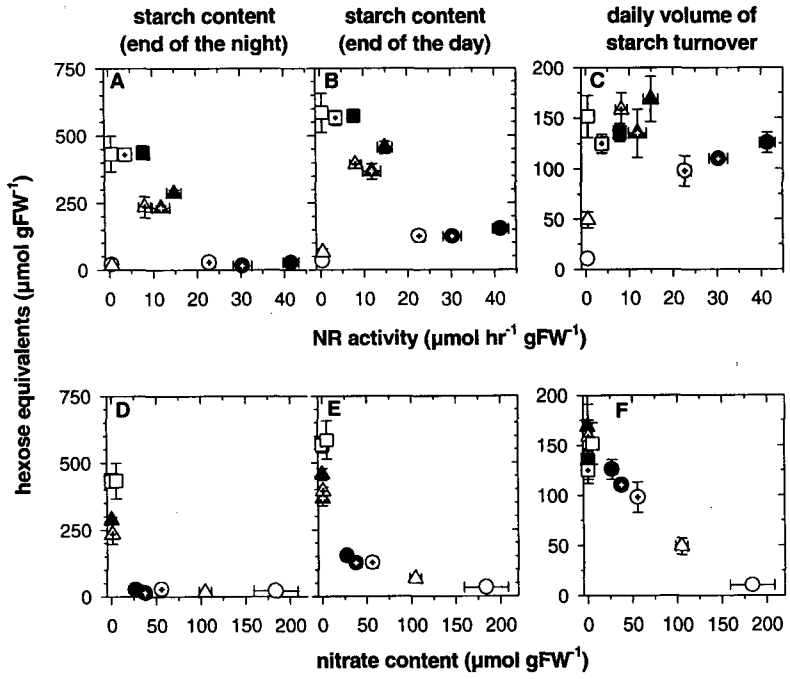

Figure 8. Diurnal Changes of Starch in Source Leaves.

Wild-type plants (filled symbols), F22×F23 and F23×Nia30 (filled and open cross-haired symbols, respectively), and Nia30(145) (open symbols) were grown on 12 (circles), 1.6 (triangles), or 0.2 (squares) mM nitrate in a 12-hr-light/12-hr-dark cycle. The plants were used when they had two to three fully expanded leaves but before stem elongation started. Wild-type plants and F22×F23 and F23×Nia30 grown on 12 mM nitrate were 32 to 35 days old; wild-type plants and F22×F23 and F23×Nia30 grown on 1.6 mM nitrate were 40 to 42 days old; wild-type plants and F23×Nia30 grown on 0.2 mM nitrate and Nia30(145) grown on 12 mM nitrate were 60 to 62 days old; Nia30(145) grown on 1.6 mM nitrate was 70 days old; and Nia30(145) grown on 0.2 mM nitrate was 90 days old. Samples were removed from the youngest fully expanded leaf during the last 20 min of the dark period (**[A]** and **[D]**) and the last 20 min of the light period (**[B]** and **[E]**) and analyzed for starch, NR activity, and nitrate. The rate of starch synthesis (**[C]** and **[F]**) is calculated as the difference between the starch content at the end of the night and at the end of the day. The results are the mean ± SE of four or five separate plants. FW, fresh weight.

(A) Starch content at the end of the night compared with NR activity.

(B) Starch content at the end of the day compared with NR activity.

(C) The rate of starch synthesis compared with NR activity.

(D) The relationship between the contents of nitrate and starch in leaves at the end of the night.

(E) The relationship between the contents of nitrate and starch in leaves at the end of the day.

(F) The relationship between the leaf nitrate content and the rate of starch synthesis.

transcript levels before the total concentration of ions had been increased significantly.

Induction of Nitrate and Ammonium Assimilation

As previously seen (Pouteau et al., 1989; Cheng et al., 1991; Gowri et al., 1992; Kronenberger et al., 1993; Wray, 1993; Lin et al., 1994), nitrate induces genes that encode proteins that are required to reduce nitrate to ammonium, leading to an increase in the *nia* and *nii* transcripts, NR protein, and NiR activity in leaves and roots. Nitrate also induces genes that are required for ammonium assimilation. The *gln1* and *glu* transcript levels were low in the leaves and roots of wild-type plants grown on low nitrate and high when Nia30(145) was grown on high nitrate. There are multiple genes for *gln1* in many species, including tobacco (Dubois et al., 1996; Lam et al., 1996). The appearance of a new *gln1* transcript in Nia30(145) (Figure 3) indicates that nitrate may induce a specific GS1 in tobacco. One of two *gln1* genes is also induced after adding nitrate to maize (Sukanya et al., 1994). The *gln2* transcript only increased slightly in Nia30(145) compared with the wild type (see Figure 3). The plastidic GS2 accounts for most of the GS activity in the leaf (Lam et al., 1996), which may explain why total GS activity did not increase in the leaves of Nia30(145) on 12 mM nitrate. The

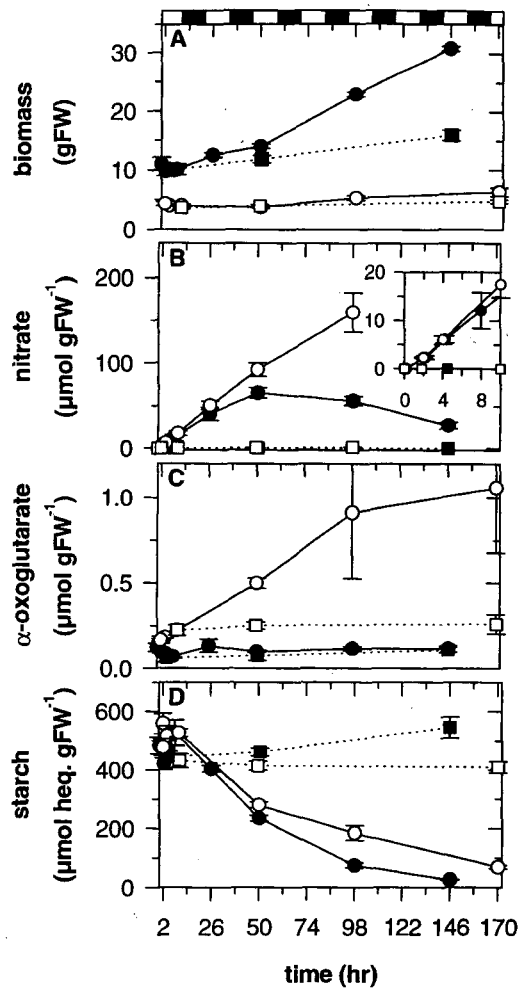

Figure 9. Changes in Growth, Nitrate, α-Oxoglutarate, and Starch Content after Resupplying High Nitrate to Nitrate-Depleted Wild-Type Plants and Severely NR-Deficient Transformants.

Results are shown for wild-type plants (filled symbols) and Nia30(145) (open symbols). Plants were grown on low nitrate (a daily addition of 0.2 mM nitrate 2 hr into the photoperiod) in a 12-hr-light/12-hr-dark cycle for 9 weeks, by which time they had two to three fully expanded leaves. The experiment was initiated by adding 12 mM nitrate 2 hr into the photoperiod. Samples (circles) were taken from the first fully expanded leaves just before nitrate addition and 2, 4, and 8 to 10 hr later. On subsequent days, plants were also supplied with 12 mM nitrate after 2 hr of illumination, and samples (circles) were taken 2 hr later. Control plants (squares), which were watered with 0.2 mM nitrate after 2 hr of illumination, were also sampled. The results are related to the fresh weight (FW) and are the mean ± SE of four separate plants.

(A) Total plant fresh weight.

(B) Nitrate content in the leaves.

(C) α-Oxoglutarate content in the leaves.

(D) Starch content in the leaves (given as micromoles of hexose equivalents [heq.] per gram fresh weight).

increase of the *gln1* transcript led to a large increase in GS activity in the roots (Table 1).

Induction of Organic Acid Synthesis

During nitrate assimilation, α-oxoglutarate is required as an acceptor for ammonium in the GOGAT pathway, and malate and citrate are required as counteranions to replace nitrate and prevent alkalinization. Malate is synthesized from PEP via PEPcase, and citrate is synthesized by a concerted action of PEPcase, pyruvate kinase, pyruvate dehydrogenase, and citrate synthase (Figure 1). The conversion of isocitrate to α-oxoglutarate occurs in the cytosol and is catalyzed by the NADP-ICDH dehydrogenase (Figure 1; Hanning and Heldt, 1993; Fieuw et al., 1995).

Nitrate-deficient wild-type plants had low levels of the *ppc* transcript, low PEPcase activity, low levels of malate and citrate, and small pools of isocitrate and α-oxoglutarate. In contrast, Nia30(145) transformants grown on 12 mM nitrate had high *ppc* transcript levels and also contained enhanced transcript levels of other genes required for the synthesis of organic acids, including pk_c, cs_m, and *icdh1*. All of these transcripts showed marked changes in response to relatively small changes of nitrate in mutants with moderate changes in NR activity, and the *ppc* transcript increased within 2 hr when nitrate was added to nitrate-deficient wild-type plants or Nia30(145). These results show that nitrate initiates a coordinated increase in the expression of several genes involved in organic acid synthesis. The changes in expression lead to marked changes in metabolism. Nia30(145) grown on 12 mM nitrate contained high PEPcase activity, accumulated substantial amounts of malate and citrate, and contained extremely large pools of isocitrate and α-oxoglutarate. Metabolism was affected in the leaves and the roots, with a particularly large accumulation of malate and citrate in the latter.

Signals derived from nitrate may also modify the post-translational regulation of PEPcase. PEPcase extracted from nitrate-deficient wild-type plants is very sensitive to inhibition by malate, indicating that it has been inactivated by dephosphorylation (see also Duff and Chollet, 1995). In contrast, PEPcase from Nia30(145) plants grown in high nitrate is much less sensitive to malate. Further studies are needed to investigate the effects of nitrate on the activity of PEPcase kinase.

In wild-type plants, nitrate stimulates the synthesis of organic acids, which are used in amino acid synthesis or stored as counteranions. In Nia30(145), nitrate stimulates the synthesis of organic acids, but organic acids accumulate because nitrate assimilation is restricted by the low NR activity. The extent to which organic acids accumulate presumably depends on the sensitivity of PEPcase to feedback inhibition by organic acids and on the rate at which organic acids are metabolized or exported from the tissue. Organic acids are exported from the leaves to the roots (Imsande and Touraine, 1994), which may explain why malate and citrate do not accumulate to such high levels in the leaves.

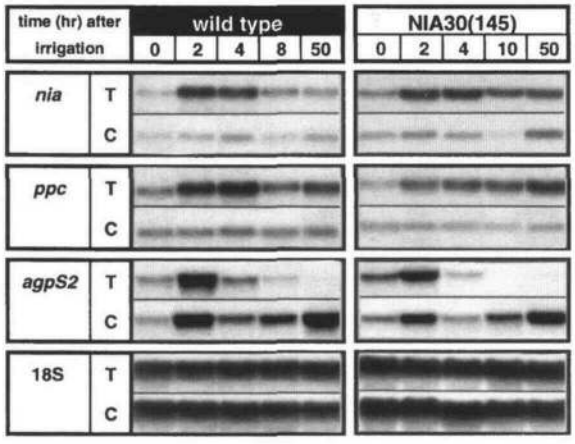

Figure 10. Changes of *nia*, *ppc*, and *agpS2* Transcripts after Resupplying High Nitrate to Nitrate-Depleted Wild-Type Plants and Severely NR-Deficient Transformants.

Plants had been grown on 0.2 mM nitrate provided each day after 2 hr of illumination up to the day on which the experiment began. On this day, control plants were provided with 0.2 mM nitrate as usual (C) or irrigated with 12 mM nitrate nutrient solution after 2 hr of illumination (T). See the legend to Figure 9 for further details of the experiment design and sampling.

Repression of Starch Synthesis

Numerous studies have found a striking negative relationship between nitrate fertilization and starch levels (Hofstra et al., 1985; Waring et al., 1985; Fichtner and Schulze, 1992; Stitt and Schulze, 1994; see also Figures 8D and 8E). By accumulating starch, slow-growing plants can store carbon in an osmotically inactive form (Hehl and Mengel, 1972; Radin and Eidenbock, 1986; Rufty et al., 1988). AGPase plays a key role in the regulation of starch synthesis (Preiss et al., 1991). Higher plant AGPase is a heterodimer consisting of a regulatory and catalytic subunit (encoded by *agpS* and *agpB*, respectively). Expression of *agpS2* (which encodes the regulatory subunit of leaf AGPase) is increased by sugars (Müller-Röber et al., 1990), and AGPase is allosterically regulated by the 3PGA/Pi ratio (Preiss et al., 1991). However, until now, no mechanisms were known that could directly link starch and nitrogen metabolism.

Our results pinpoint two ways in which starch synthesis is regulated by nitrate. First, nitrate inhibits expression of *agpS2*. Nitrate-deficient wild-type plants normally have high levels of the *agpS2* transcript and high AGPase activity relative to PEPcase and SPS. In contrast, Nia30(145) grown on 12 mM nitrate contains low levels of the *agpS2* transcript and low AGPase activity. The *agpS2* transcript decreases within 4 hr of adding nitrate to nitrate-deficient wild-type plants or Nia30(145) grown previously on low nitrate, and this decrease is faster than and independent of the changes in nitrate assimilation or plant growth. Second, nitrate-deficient wild-types have relatively high levels of 3PGA. Nitrate leads to increased activity of PEPcase and decreases the concentration of this allosteric activator of AGPase.

The importance of nitrate as a signal to regulate starch metabolism is highlighted in Nia30(145). When nitrate accumulates in Nia30(145), starch metabolism becomes uncoupled from the rate of growth. Nia30(145) plants synthesize negligible amounts of starch even though they have very low growth rates, and after adding nitrate back to nitrate-deficient plants, starch was remobilized almost as rapidly in Nia30(145) as in wild-type plants, even though growth was not increased in Nia30(145) (Figure 9). The latter result indicates that nitrate may also stimulate starch degradation.

The accumulation of nitrate did not lead to a parallel inhibition of sucrose synthesis. Compared with nitrate-deficient wild-type plants, Nia30(145) grown on 12 mM nitrate had slightly higher transcript for *sps*, approximately two times higher SPS activity, no post-translational inactivation of SPS, and unaltered or even higher levels of sucrose and reducing sugars in their leaves. The selective inhibition of starch accumulation by nitrate has interesting physiological consequences. First, sucrose constitutes the major osmoticum in the phloem. Continued synthesis and export of sucrose

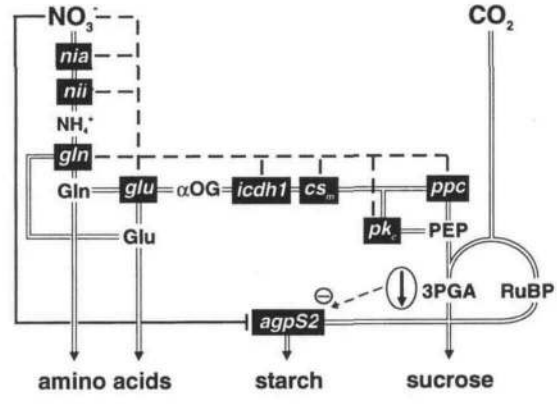

Figure 11. Coordinated Regulation of Carbon and Nitrogen Metabolism by Nitrate.

The scheme shows the effect of nitrate on the expression of genes (filled boxes) encoding enzymes in the metabolic pathways (open double line) involved in nitrate assimilation and the synthesis of amino acids, starch, and sucrose. See the legend to Figure 1 for the abbreviations of the metabolic intermediates. Induction is shown as dashed lines and repression of *agpS2* as a solid line. A mechanism is also indicated that allows further indirect inhibition of AGPase (dashed arrow) involving increased expression and activity of PEPcase and a resulting decrease in the allosteric activator 3PGA. The circled arrow represents a decrease of 3PGA after increased expression of *ppc*.

during nitrate assimilation therefore allows a high turgor to be maintained in the phloem and promotes the export of the amino acids from the leaves. Second, continued synthesis and export of sucrose to the growing tissues promote rapid use of the amino acids. This interdependence between sucrose synthesis and the export and use of amino acids may explain why higher plants differ from algae, in which nitrate assimilation leads to a general inhibition of carbohydrate synthesis (Huppe and Turpin, 1994).

Nitrate Initiates a Program of Gene Expression Leading to Major Changes in Metabolism and Growth

In conclusion, nitrate is one of the compounds that the plant measures to adjust metabolism and growth to changes in the availability of nitrogen. Earlier studies by other investigators have shown that genes required for nitrate uptake and reduction are induced by nitrate. The results obtained in this study reveal that nitrate initiates a more extended program of gene expression (Figure 11), resulting in coordinate alterations in the activities of enzymes in several metabolic pathways that are directly or indirectly involved in the further metabolization and use of nitrate, including ammonium assimilation, organic acid synthesis, and starch metabolism. In a separate report (Scheible et al., 1997a), we showed that the accumulation of nitrate in tobacco shoots initiates changes in shoot–root allocation.

It is likely that further processes are also regulated by nitrate. Nitrate uptake and assimilation require energization of the plasmalemma and regulation of the pH in the cytosol. Recent studies indicate that there is a close interaction between the regulation of nitrate metabolism and the plasmalemma ATPase (Moorhead et al., 1996). The assimilation of nitrate in nonphotosynthetic tissues requires reducing equivalents. After the addition of nitrate, there is a rapid and cycloheximide-insensitive increase in the transcript for ferredoxin–NADP–oxidoreductase in maize roots (Ritchie et al., 1994) and, in contrast to ammonium, which was ineffective, an increase in the activities of several enzymes of the oxidative pentose phosphate pathway in algae (Huppe and Turpin, 1994). The finding that the cytosolic concentration of nitrate is maintained as a constant and that surplus nitrate is accumulated in the vacuole (Walker et al., 1995) implies that nitrate regulates the activity of transport systems on the tonoplast. The genotypes used in this study should provide a useful system to investigate the role of nitrate in the regulation of these and other processes, including the transport and storage of nitrogenous compounds as well as flowering and senescence.

Further studies are needed to identify the mechanism(s) by which nitrate is sensed. The results presented here show that nitrate sensing does not require further metabolism by NR, and Pouteau et al. (1989) showed that it does not even require the presence of NR protein. Our results provide a range of phenotypic changes that might be exploited to obtain mutants in nitrate signaling. It is also important to consider whether nitrate interacts with signals derived from other compounds in carbon and nitrogen metabolism. Many of the genes regulated by nitrate are also affected by sugars, including *nia*, *icdh1*, pk_c, *ppc*, and *agpS2* (reviewed in Koch, 1996). Changes in the levels of nitrate affect the levels of sugars and vice versa, and there also may be cross-talk between the transduction pathways. Glutamine and ammonium have been implicated in the feedback repression of nitrate uptake and assimilation (Hoff et al., 1994; Quesada et al., 1997) and in the induction of the enzymes that are required for ammonium assimilation and organic acid synthesis (Sugiharto and Sugiyama, 1992; Hoff et al., 1994; Lam et al., 1996). Signals derived from nitrate presumably interact with signals derived from these nitrogen metabolites. Whereas they act antagonistically on nitrate uptake and reduction, they may act in concert on events farther downstream. Nevertheless, the results in this study and Scheible et al. (1997a) show that signals derived from nitrate can activate organic acid metabolism, repress starch synthesis, and inhibit root growth, even when plants are severely deficient in organic nitrogen.

METHODS

Plant Material and Growth

All tobacco (*Nicotiana tabacum*) genotypes were grown in a growth chamber, as previously described (Scheible et al. 1997a, 1997b). Inorganic nitrogen was provided as a mixture of 4 mM potassium nitrate and 4 mM magnesium nitrate. When nitrate was decreased to 0.2 mM, it was replaced by a mixture of potassium and magnesium chloride and sulphate to maintain the same concentration of each cation and the same overall anion concentration. Plants were harvested in the exponential phase of growth with a rosette of leaves, after 32 to 35 days for the 12 mM nitrate-fed wild-type plants and mutants, after 60 to 65 days for nitrate-limited wild-type plants and 12 mM nitrate-fed transformants, and after 85 to 91 days for 0.2 mM nitrate-fed transformants. Unless stated otherwise, samples were taken after 4 hr of illumination from the youngest fully expanded leaf. Material was harvested into liquid nitrogen and stored at −80°C. The relative growth rate was calculated as described previously (Scheible et al., 1997a).

Metabolite Analysis

Sucrose, glucose, fructose, starch, nitrate, and amino acids were measured in the soluble and residual fractions of an ethanol–water extract (Scheible et al., 1997b), as described in Stitt et al. (1989), Gebauer et al. (1984), and Geigenberger et al. (1996). Pyruvate, phospho*enol*pyruvate (PEP), glycerate 3-phosphate (3PGA), α-oxoglutarate, isocitrate, citrate, and malate were measured in perchloric acid extracts (Bergmeyer, 1989; Stitt et al., 1989). Total protein content was determined by two different methods, both described in Scheible et al. (1997a).

Enzyme Assays

Nitrate reductase (NR) activity and NR protein were measured as described in Scheible et al. (1997a, 1997b). Nitrite reductase (NiR) activity was assayed essentially as described by Wray and Fido (1990) by using dithionite-reduced methyl viologen as an artifical electron donor. Plant material stored at −80°C was ground to a fine powder in a mortar that had been precooled with liquid nitrogen, and then the powder was thoroughly mixed with and extracted in four volumes (v/w) of buffer containing 100 mM Hepes-KOH, pH 7.5, 5 mM magnesium acetate, 2 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM 6-aminocaproic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) PVP, and 0.1% (v/v) Triton X-100. The assay mixture contained 75 mM Hepes-KOH, pH 7.5, 1.2 mM $NaNO_2$, 2.4 mM methyl viologen, 15 mM $Na_2S_2O_4$, and 20 to 40 μL of enzyme extract in a final volume of 1 mL. The reaction was started by the addition of the enzyme. After various times at 25°C, 20-μL aliquots were removed from the assay mixture, mixed with 380 μL of double-distilled water, and added to 300 μL of 1% (w/v) sulfanilamide in 3 N HCl plus 300 μL of 0.01% (w/v) *N*-(1-naphthyl)ethylenediamine in double-distilled water. Tubes were allowed to stand for 20 min and then centrifuged for 5 min at 16,000*g*. The absorbance of the azodye was measured at 540 nm. Absorbances were plotted against incubation times, and NiR activity was calculated from the slope of the regression line by reference to a previously established standard plot (0 to 30 μM nitrite).

To measure glutamine synthetase (GS) activity, plant material was extracted as described above, centrifuged for 2 min at 16,000*g*, and desalted in Sephadex G25 spin columns that had been preequilibrated in extraction buffer without PMSF, Triton X-100, and PVP. GS activity was measured by a coupled spectrophotometric assay (Lea et al., 1990). The reaction mixture contained 50 mM Hepes-KOH, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.2 mM NADH, 1 mM PEP, 5 mM ATP, 0.2 mM $NaVO_3$, 40 μM 5′, 5′-diadenosine pentaphosphate, 15 μL of eluate, 5 units of pyruvate kinase, and 3 units of lactate dehydrogenase (both added as glycerol solution; Boehringer Mannheim) in a final volume of 900 μL. The rates of NADH oxidation at 340 nm and 25°C were followed before and after addition of 15 mM glutamate and 1 mM NH_4Cl, and the difference was used to calculate the activity. Controls were performed with eluants preincubated on ice for 15 min with or without 10 mM methionine sulfoxime to show that the increased rates of NADH oxidation after substrate addition were due to GS activity.

To measure PEP carboxylase (PEPcase) activity, plant material was extracted as described above in a buffer containing 100 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 10% (w/v) sorbitol, 1 mM EDTA, 5 mM DTT, 1 mM NaF, 1% (w/v) PVP, 1 mM PMSF, 1 mM benzamidine, and 1 mM 6-aminocaproic acid. The extract was centrifuged, desalted rapidly on a Sephadex G25 column equilibrated in extraction buffer without PMSF, PVP, and sorbitol, and used immediately for activity determination. PEPcase activity was measured spectrophotometrically by following the rate of PEP-dependent NADH oxidation at 340 nm and 25°C by coupling to exogenous malate dehydrogenase. The 800-μL reaction mixture contained 50 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 5 mM $NaHCO_3$, 0.2 mM NADH, 4 units of malate dehydrogenase (from porcine heart mitochondria), and 20 to 40 μL of desalted extract with or without 5 mM L-malate. The reaction was started by addition of 2.5 mM PEP, and the PEPcase activity was calculated from the increase in the NADH oxidation rate.

AGPase activity was measured in extracts that were made as described above, with a buffer containing 50 mM Hepes-KOH, pH 7.4, 5 mM $MgCl_2$, 10% glycerol, 1% PVP, 0.1% Triton X-100, and EDTA, EGTA, DTT, PMSF, benzamidine, and 6-aminocaproic acid at 1 mM each. After centrifugation (5 min at 16,000*g*), the supernatant was immediately used for activity tests. Production of glucose 1-phosphate from ADP-glucose was determined in a NAD-linked glucose-6-phosphate dehydrogenase system (Smith, 1990). The 0.6-mL reaction mixture contained 50 mM Hepes-KOH, pH 7.8, 5 mM $MgCl_2$, 1 mM NAD, 1 mM NaPPi, 1 unit of phosphoglucomutase, 1 unit of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 10 to 20 μL of extract. NAD reduction was monitored spectrophotometrically at 334 nm and 25°C before and after the addition of 2 mM ADP–glucose by using a dual wavelength photometer (model ZFP22; Sigma).

SPS activity was measured under V_{max} (with 12 mM fructose 6-phosphate, 36 mM glucose 6-phosphate, and 6 mM UDP glucose) and V_{sel} conditions, including limiting substrate concentrations and the inhibitor phosphate (with 2 mM fructose 6-phosphate, 6 mM glucose 6-phosphate, 6 mM UDP-glucose, and 5 mM phosphate) to detect total activity and the activity of the active dephosphorylated form, as described by Reimholz et al. (1997).

RNA Gel Blot Analysis

Total RNA was isolated from roots or leaf material from which the first-, second-, and third-order veins had been removed, and RNA gel blot analyses were performed with 20 μg of total RNA per lane, as described in Krapp et al. (1993), except that the RNA was covalently fixed to the blotting membrane (Hybond N; Amersham) by UV crosslinking (Stratalinker 1800; Stratagene, La Jolla, CA). The radioactively hybridized filters were washed with increasing stringency and, depending on the probe used, until the signal/background ratio was maximal. Autoradiography was performed at −80°C with Kodak X-Omat films with intensifying screens. Hybridized RNA gel blots were stripped by washing up to three times for 1 min in 0.1% (w/v) SDS at 90°C. The quantitative removal of the radiolabeled cDNA was checked by autoradiography before using the RNA membranes for another hybridization.

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