

Regulation of Root Ion Transporters by Photosynthesis: Functional Importance and Relation with Hexokinase

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Coordination between the activity of ion transport systems in the root and photosynthesis in the shoot is a main feature of the integration of ion uptake in the whole plant. However, the mechanisms that ensure this coordination are largely unknown at the molecular level. Here, we show that the expression of five genes that encode root NO_3^- , NH_4^+ , and SO_4^{2-} transporters in *Arabidopsis* is regulated diurnally and stimulated by sugar supply. We also provide evidence that one Pi and one K^+ transporter also are sugar inducible. Sucrose, glucose, and fructose are able to induce expression of the ion transporter genes but not of the carboxylic acids malate and 2-oxoglutarate. For most genes investigated, induction by light and induction by sucrose are strongly correlated, indicating that they reflect the same regulatory mechanism (i.e., stimulation by photosynthates). The functional importance of this control is highlighted by the phenotype of the *atnrt2* mutant of *Arabidopsis*. In this mutant, the deletion of the sugar-inducible NO_3^- transporter gene *AtNrt2.1* is associated with the loss of the regulation of high-affinity root NO_3^- influx by light and sugar. None of the sugar analogs used (3-O-methylglucose, 2-deoxyglucose, and mannose) is able to mimic the inducing effect of sugars. In addition, none of the sugar-sensing mutants investigated (*rsr1-1*, *sun6*, and *gin1-1*) is altered in the regulation of *AtNrt2.1* expression. These results indicate that the induction of *AtNrt2.1* expression by sugars is unrelated to the main signaling mechanisms documented for sugar sensing in plants, such as regulation by sucrose, hexose transport, and hexokinase (HXK) sensing activity. However, the stimulation of *AtNrt2.1* transcript accumulation by sucrose and glucose is abolished in an antisense *AtHXK1* line, suggesting that HXK catalytic activity and carbon metabolism downstream of the HXK step are crucial for the sugar regulation of *AtNrt2.1* expression.

INTRODUCTION

As a specialized function of root cells, the uptake of mineral ions has to be integrated in the plant to match the nutrient demand of the whole organism. This integration is ensured by regulatory mechanisms that modulate the expression and/or the activity of root ion transport systems according to the nutritional status of the plant. Specific feedback downregulation of root ion transporters by the ions themselves, or the products of their metabolism, probably plays a central role in this context (Grignon, 1990; Clarkson and Lüttge, 1991; Chrispeels et al., 1999). However, a more general control over ion uptake also has been documented that coordinates the activity of root transport systems with the photosynthetic activity of the shoot (Forde, 2002). Uptake rates of many ions are dependent on light conditions and fluctuate diurnally (Clément et al., 1978;

Smith and Cheema, 1985; Hatch et al., 1986; Le Bot and Kirkby, 1992; Delhon et al., 1995a) or are stimulated by an increase in light intensity (Gastal and Saugier, 1989).

On a functional basis, the regulation of root ion transport by photosynthesis has been documented particularly in the case of NO_3^- , the uptake of which is regulated by the CO_2 concentration in the atmosphere (Gastal and Saugier, 1989; Delhon et al., 1996), and by the availability in the roots of various C metabolites, such as sugars (Hänisch Ten Cate and Breteler, 1981; Ruffy et al., 1989; Delhon et al., 1996) and carboxylic acids (Touraine et al., 1992). Furthermore, root NO_3^- uptake is almost independent of the variations in transpiration and is not under the control of an endogenous circadian rhythm (Delhon et al., 1995b). Collectively, these observations have led to the proposal that the light control of NO_3^- uptake reflects a regulation exerted by the downward transport of photosynthates (Delhon et al., 1996). As such, this regulation is part of a general framework of reciprocal controls between N and C metabolisms that ensures their coordination at the whole-plant level (Lam et al., 1996; Stitt, 1999; Stitt and Krapp, 1999; Coruzzi and Zhou, 2001).

Despite clear evidence for the positive action of sugars on NO_3^- uptake, very few studies have been conducted at the molecular level to investigate the mechanisms involved. However,

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the bases for the signaling role of sugars have been determined by studies of enzymes of N assimilation. For instance, nitrate reductase (NR), Gln synthetase, and Asn synthetase were shown to be regulated by sugars at the transcriptional level (Cheng et al., 1992; Lam et al., 1996). Moreover, a powerful mechanism of post-translational regulation has been described for both NR (Kaiser and Huber, 1994) and Gln synthetase (Finnemann and Schjoerring, 2000) that inactivates the two enzymes after the light/dark transition or under conditions in which photosynthesis is impaired. Sugars are able to prevent this inactivation in the case of NR (De Cires et al., 1993) and thus are considered putative signals involved in the tuning of amino acid synthesis as a function of current carbon assimilation in the leaves. The crosstalk between N and C metabolisms also is illustrated by the reverse control (i.e., regulation of C assimilation and utilization by N compounds). As an example, NO_3^- has been shown to act as a signal regulating the expression of the genes that encode phosphoenolpyruvate carboxylase, cytosolic pyruvate kinase, citrate synthase, and NADP isocitrate dehydrogenase (Scheible et al., 1997; Stitt, 1999).

In our previous study with *Arabidopsis* (Lejay et al., 1999), we found that *AtNrt1.1* and *AtNrt2.1*, which encode NO_3^- transporters, are regulated diurnally in the roots. Both genes display an increase in transcript accumulation during the light period followed by a decrease during the night. Supply of sucrose to the roots at the beginning of the night prevents the decline in *AtNrt1.1* and *AtNrt2.1* expression, indicating that these genes are sugar inducible and that sucrose could mimic the effect of light on their expression. Because the variations in *AtNrt1.1* and *AtNrt2.1* transcript accumulation paralleled those of NO_3^- influx, we proposed that the coordination of root NO_3^- uptake with photosynthesis may rely on the sugar regulation of root NO_3^- transporters (Lejay et al., 1999). Three genes that encode high-affinity NH_4^+ transporters (*AtAmt1.1*, *AtAmt1.2*, and *AtAmt1.3*) also were shown to display diurnal changes in root expression similar to those of *AtNrt1.1* and *AtNrt2.1* (Gazzarrini et al., 1999), suggesting that they may be regulated by sugars as well (von Wirén et al., 2000a). After these observations were made in *Arabidopsis*, both NO_3^- and NH_4^+ transporter genes were found to be regulated by the light/dark transition and photosynthesis in tomato (Ono et al., 2000; von Wirén et al., 2000b) and tobacco (Matt et al., 2001). In addition, K^+ channel genes also were reported to be induced by light, photosynthesis, and sugars in the shoots of both *Arabidopsis* and *Vicia faba* (Deeken et al., 2000; Ache et al., 2001). Thus, evidence is emerging that the sugar regulation of ion transporter gene expression is a widespread mechanism, allowing the coordination of the transport of various ions with photosynthesis and the C status of the plant. However, in spite of the considerable efforts undertaken to elucidate the complex mechanisms involved in the sugar regulation of gene expression (Sheen et al., 1999; Gibson, 2000; Smeekens, 2000; Coruzzi and Zhou, 2001; Rolland et al., 2002), very little information is available concerning ion transporters.

The aim of this work was to further investigate the regulation of ion transporter genes by light and sugars. To determine the generality of the observations made regarding the NO_3^- transporters, a larger set of genes was studied, including the three *AtAmt1* NH_4^+ transporter genes known to be regulated diurnally

as well as genes that encode transporters or channels for other ions than NO_3^- or NH_4^+ . In fact, a significant proportion of genes regulated diurnally in *Arabidopsis* are controlled by an endogenous circadian rhythm (Schaffer et al., 2001). Thus, special attention was given in our study to determine whether light/dark changes in the expression of root ion transporters actually are related to regulation by photosynthesis and sugars. Most importantly, the functional significance of this regulation was investigated, in particular through the analysis of the *atnrt2* mutant of *Arabidopsis*, which has a deletion of *AtNrt1.1* and *AtNrt2.2* (Filleur et al., 2001). Finally, the search for the regulatory mechanisms was initiated by testing three of the main transduction pathways proposed to be involved in the sugar regulation of gene expression: signaling by sucrose itself (Chiou and Bush, 1998), by hexose transport (Martin et al., 1997), and by HXK (Jang et al., 1997).

RESULTS

Light Stimulation and Sucrose Induction of the Expression of Root Ion Transporter Genes Are Strongly Correlated

To allow a more complete identification of the root ion transporters regulated by light and sugars, we extended our study to the expression of 13 genes that encode ion transporters or channels. These genes, which are expressed significantly or predominantly in the roots, include (in addition to *AtNrt1.1* and *AtNrt2.1*) *AtAmt1.1*, *AtAmt1.2*, and *AtAmt1.3*, which encode high-affinity NH_4^+ transporters (Gazzarrini et al., 1999); *AtHst1*, which encodes a high-affinity SO_4^{2-} transporter (Vidmar et al., 2000); *AtPt1* and *AtPt2*, which encode high-affinity phosphate transporters (Muchhal et al., 1996); *AtAkt1* and *AtSkor*, which encode K^+ channels (Sentenac et al., 1992; Gaymard et al., 1998); *AtKup2*, which encodes a high-affinity K^+ transporter (Kim et al., 1998); and *AtIrt1* and *AtNramp1*, which encode metal transporters (Eide et al., 1996; Curie et al., 2000).

First, we investigated the regulation of both NH_4^+ transport and transporters, because the expression of three *AtAmt1* genes was found previously to be regulated diurnally (Gazzarrini et al., 1999). Influx of NH_4^+ in *Arabidopsis* roots showed marked diurnal changes, with a fast and strong decline after the light-to-dark transition (Figure 1A). Supply of 1% sucrose to the nutrient solution at the beginning of the dark period prevented this decline and led to an almost unchanged root NH_4^+ influx during the night compared with that at the end of the day (Figure 1A). In addition to being regulated diurnally in a manner similar to NH_4^+ influx, all three *AtAmt1* genes were induced by sucrose supply in the dark (Figure 1B). However, both diurnal variations and response to sucrose were much more pronounced for *AtAmt1.2* and *AtAmt1.3* than for *AtAmt1.1*.

The changes in expression observed for the eight other genes indicated that several other transporters also were induced by light and/or sucrose at the transcript level (Figures 2 and 3). *AtHst1* displayed an increase in transcript accumulation during the photoperiod and induction by sucrose. The expression of *AtPt2* and *AtKup2* showed less pronounced diurnal changes but clearly was induced by sucrose supply (Figures 2 and 3). *AtIrt1* also was regulated diurnally, but with changes in

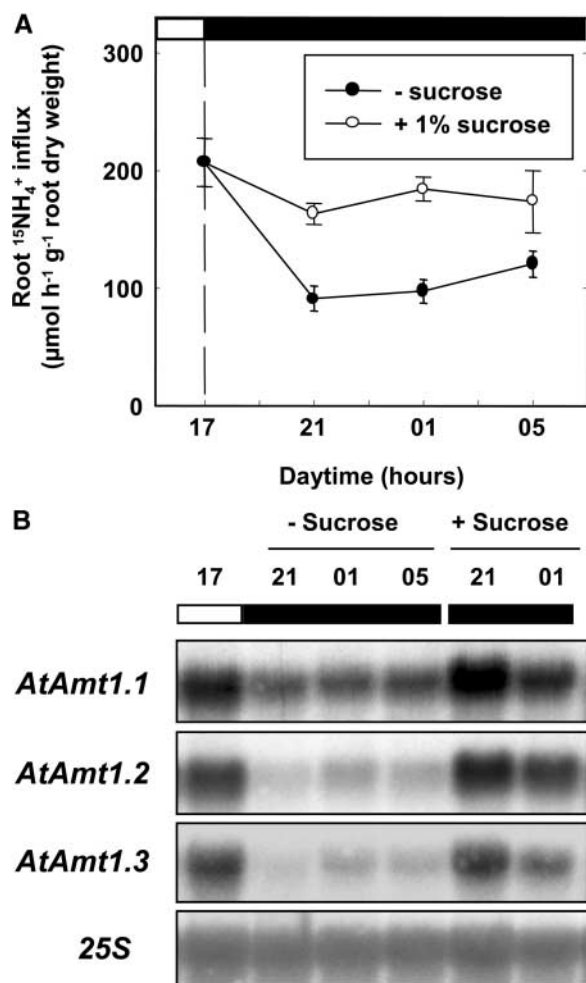


Figure 1. Sucrose Stimulation of Root Ammonium Uptake and Expression of Ammonium Transporter Genes in the Roots.

(A) Time course of root $^{15}\text{NH}_4^+$ influx during the dark period of the day/night cycle in plants supplied with a complete nutrient solution containing 1 mM NH_4NO_3 with or without 1% sucrose. The plants were 6 weeks old and were grown under an 8-h-day/16-h-night cycle from germination. The sucrose treatment was initiated at the beginning of the dark period, with 50 mg/L penicillin and 25 mg/L chloramphenicol added to nutrient solutions of both treated (+1% sucrose) and control (–sucrose) plants. Root $^{15}\text{NH}_4^+$ influx was assayed at 200 μM external $^{15}\text{NH}_4^+$ concentration, and the values shown are means of 12 replicates \pm SE. The white and black boxes at top indicate light and dark periods, respectively.

(B) RNA gel blot analysis of the expression of *AtAmt1.1*, *AtAmt1.2*, and *AtAmt1.3* NH_4^+ transporter genes in roots during the dark period of the day/night cycle in plants supplied with a complete nutrient solution containing 1 mM NH_4NO_3 with or without 1% sucrose. The plants were from the same experiment as those used for measurements of uptake activity in **(A)**. RNA sample loading was quantified using a 25S probe.

expression not fully in phase with the light/dark transitions, as indicated by a marked increase in transcript accumulation before the end of the night (Figure 2). *AtSkor* displayed a diurnal rhythm of expression almost opposite to that of illumination,

with maximum transcript accumulation at the end of the night (Figure 2). The three other genes, *AtAkt1*, *AtNramp1*, and *AtPt1*, showed no significant changes in transcript accumulation during the day/night cycle and were not sucrose inducible (Fig-

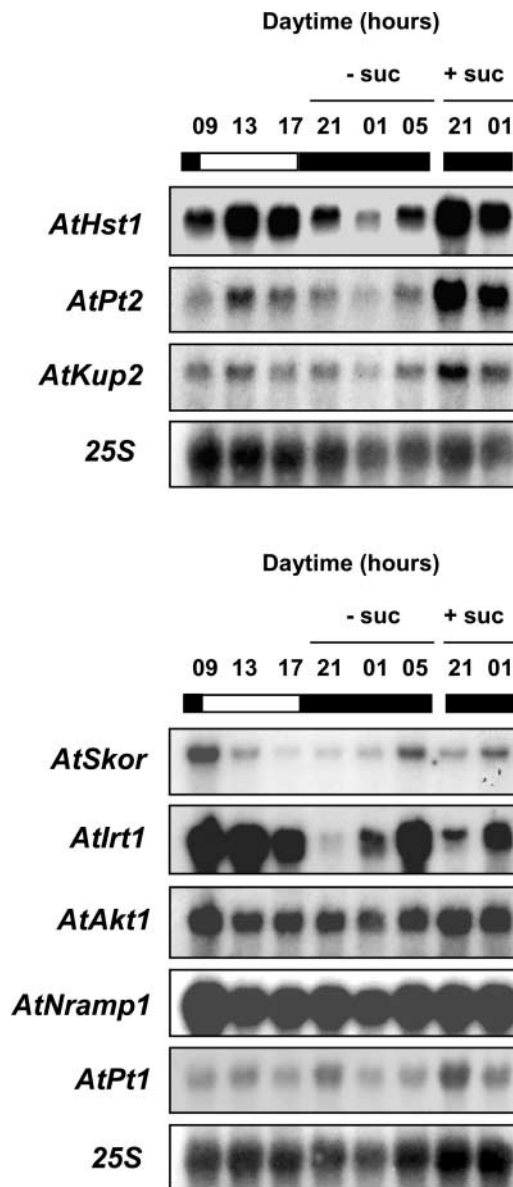


Figure 2. Diurnal Changes and Sucrose Induction of the Expression of Various Ion Transporter and Channel Genes in the Roots.

RNA gel blot analysis of *AtHst1*, *AtPt2*, *AtKup2*, *AtSkor*, *AtIrt1*, *AtAkt1*, *AtNramp1*, and *AtPt1* expression during the day/night cycle in the roots of plants supplied with or without 1% sucrose for the whole dark period. Six-week-old plants were grown on a complete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle. The sucrose treatment was initiated at the beginning of the dark period, with 50 mg/L penicillin and 25 mg/L chloramphenicol added to nutrient solutions of both treated (+suc) and control (–suc) plants. RNA sample loading was quantified using a 25S probe. The white and black boxes at top indicate light and dark periods, respectively.

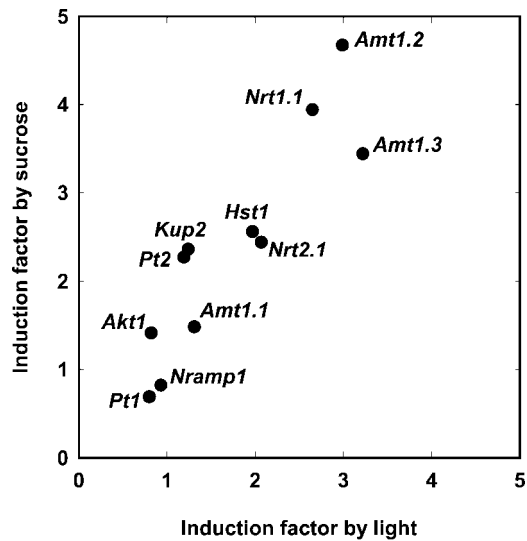


Figure 3. Correlation between the Induction of Ion Transporter and Channel Gene Expression in the Roots by Light and Sucrose Supply.

The experimental conditions were the same as those described for Figure 2. The induction factor by light was calculated for each gene as the ratio of the relative mRNA accumulation at the end of the light period (17 h) to the relative mRNA accumulation after 4 h in darkness (21 h). The induction factor by sucrose was calculated after 4 h in darkness (21 h) as the ratio of the relative mRNA accumulation in sucrose-supplied plants to that in control plants without sucrose. Relative mRNA accumulation was normalized using a 25S probe. The data shown are means from two independent experiments. Neither *AtSkor* nor *AtIrt1* was considered because of their diurnal pattern of expression out of phase with the light/dark transition.

ures 2 and 3). When the quantified changes in gene expression obtained in two independent experiments were pooled, a strong correlation was found between the stimulating effects of light and sucrose (Figure 3), showing that these two means of regulation probably are linked. Accordingly, the concentrations of soluble sugars in the roots increased during the light period (Figure 4). Figure 3 also shows that there may be no clear distinction between ion transporter genes regulated or not by light and sucrose. Rather, a continuum between genes strongly affected or not affected at all by these factors was observed.

To unambiguously demonstrate that the diurnal changes in ion transporter gene expression are not attributable to a circadian rhythm, we investigated the effect of continuous light. For *AtNrt2.1*, *AtNrt1.1*, *AtAmt1.3*, *AtHst1*, *AtKup2*, and *AtPt2*, leaving the light on during the usual night completely prevented the decrease in transcript accumulation normally associated with the dark period of the day/night cycle (Figure 5B). The same response was found for the activity of the NO_3^- uptake systems, because continuous light resulted in a strong stimulation of root NO_3^- influx, which increased to very high values during the usual period of darkness (Figure 5A). The relationship between ion transporter gene expression in the root and the C status of the plant was documented further by the strong dependence of the transcript accumulation of *AtNrt2.1*, *AtNrt1.1*, and *AtAmt1.3*

on the light intensity received by the plant canopy (Figure 6A). Increasing the light intensity also resulted in higher concentrations of soluble sugars in the roots (Figure 6B).

To test the specificity of the action of photosynthates, we investigated the effect of carbon metabolites other than sugars, with a particular attention to carboxylic acids. The reason for this is that NO_3^- , NH_4^+ , and SO_4^{2-} transporter genes were found to be particularly sensitive to regulation by light and sucrose (Figure 3). Because both N and S are incorporated into amino acids, this finding suggests that carboxylic acids, the carbon skeletons for amino acids, can be the actual regulatory compounds. However, neither malate nor 2-oxoglutarate was able to mimic the stimulating effect of sucrose on the expression of *AtNrt2.1*, *AtAmt1.2*, *AtAmt1.3*, and *AtHst1* (Figure 7A). Indeed, slightly decreased levels of mRNA were found for several of these genes after malate or 2-oxoglutarate treatment compared with the levels in control plants. Malate concentrations in the roots were modified only slightly after exogenous supply of either sucrose or malate, showing that the large variations of transporter gene expression associated with sucrose supply occurred without major changes in the internal availability of malate (cf. Figures 7A and 7B). Unlike malate, 2-oxoglutarate accumulated markedly in the roots after exogenous treatment but only slightly after sucrose addition in the nutrient medium (Figure 7B). These results show that 2-oxoglutarate was taken up readily by the root cells but suggest that this compound has no stimulatory effect on the expression of the ion transporter genes investigated.

Root Nitrate Uptake Is Uncoupled from Photosynthesis in the *atnrt2* Mutant

A key question raised by these data is whether the transporter genes regulated by the light/dark transition and sucrose supply

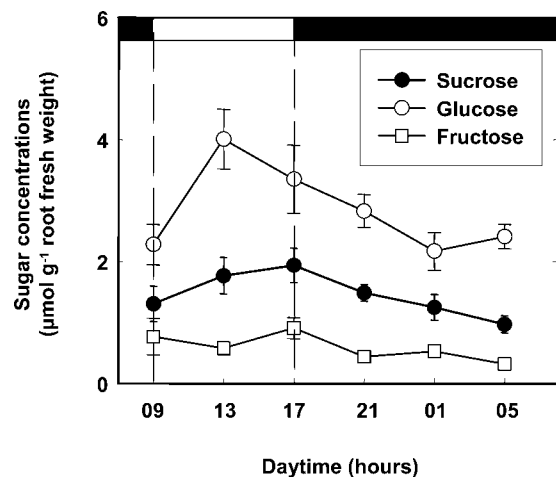


Figure 4. Diurnal Pattern of Sucrose, Glucose, and Fructose Accumulation in the Roots.

Six-week-old plants were grown on a complete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle. The data shown are means of four replicates \pm SE.

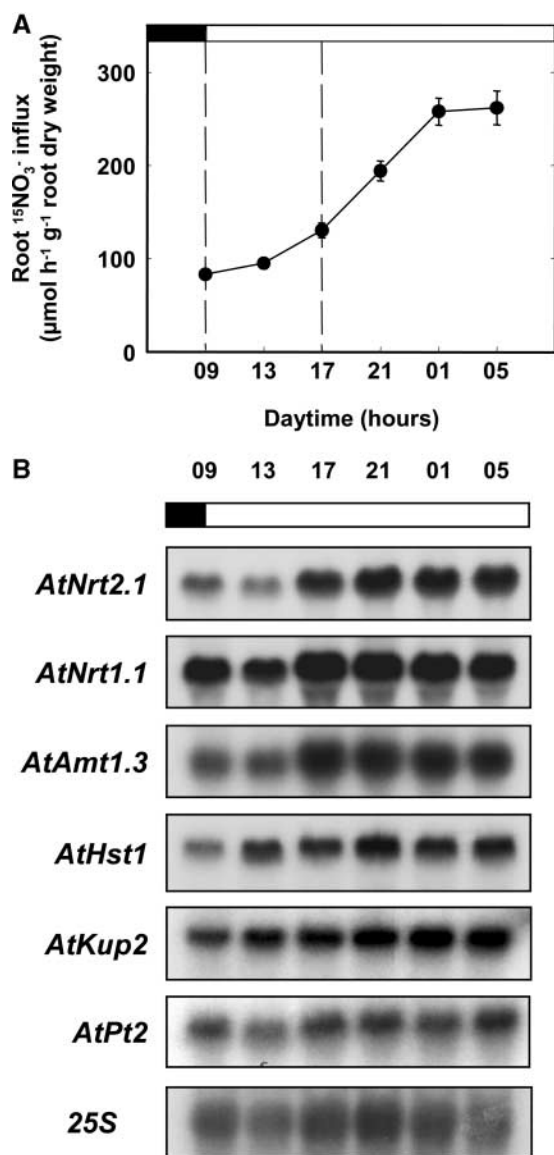


Figure 5. Effect of Continuous Light on Root Nitrate Uptake and Ion Transporter Gene Expression in the Roots.

(A) Time course of root $^{15}\text{NO}_3^-$ influx in plants left for 1 day under continuous light. Six-week-old plants were grown on a complete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle. On the day of the experiment, the light was left on for the entire usual dark period (17 h to 09 h). Root $^{15}\text{NO}_3^-$ influx was measured at 200 μM external $^{15}\text{NO}_3^-$ concentration. The values shown are means of 12 replicates \pm SE. The white and black boxes at top indicate light and dark periods, respectively.

(B) RNA gel blot analysis of the expression of *AtNrt2.1*, *AtNrt1.1*, *AtAmt1.3*, *AtHst1*, *AtKup2*, and *AtPt2* genes in the roots during the continuous light period. RNA sample loading was quantified using a 25S probe. The white and black boxes at top indicate light and dark periods, respectively.

are responsible for the diurnal changes and sugar stimulation of root ion uptake. We investigated this question using the *atnrt2* mutant, which has a deletion of both *AtNrt2.1* and *AtNrt2.2* genes and is deficient in a component of the high-affinity transport system (HATS) for NO_3^- (Filleur et al., 2001). We show here that the *atnrt2* mutant is affected in the regulation of NO_3^- uptake by light and sucrose (Figure 8). In wild-type plants, the activity of the HATS is strongly dependent on the light conditions, with $^{15}\text{NO}_3^-$ influx being at least 2 to 3 times higher during the light period than during the night (Figure 8A). In the mutant, the diurnal changes in HATS activity were suppressed strongly (Figures 8A and 8B). Additionally, in contrast to the wild type, the HATS activity in the mutant was not increased by sucrose supply at night (Figure 8B). Finally, root $^{15}\text{NO}_3^-$ influx at the end of the day was strongly dependent on the light intensity in wild-type plants but not in the *atnrt2* mutant (Figure 8C). Thus, both light and sucrose supply failed to stimulate the high-affinity $^{15}\text{NO}_3^-$ influx in the *atnrt2* mutant.

Sugar Regulation of Root Ion Transporters Is Not Related to Signaling by Sucrose, Hexose Transport, or HXK-Sensing Activity

Because of its likely central role in coordinating N and C acquisitions by the plant, *AtNrt2.1* was chosen as a model gene to further investigate the mechanisms responsible for the regulation of ion transporter gene expression by light and sugars. Sucrose does not seem to be the signal per se, because it was not the only sugar able to induce the expression of *AtNrt2.1* and to stimulate root NO_3^- uptake (Figures 9A and 9B). Exogenous supply of glucose or fructose even led to a slightly higher stimulation of *AtNrt2.1* transcript accumulation and root NO_3^- influx, compared with exogenous supply of sucrose at the same concentration. Both glucose and fructose accumulated in the roots in response to treatment with sucrose, glucose, or fructose (Figure 9C). However, sucrose concentrations in the roots increased only when sucrose was supplied exogenously.

To account for some of the effects of both sucrose and glucose on gene expression, HXK has been postulated to be a major sugar sensor, implicated in signaling pathways that govern the expression of various photosynthesis genes (Sheen et al., 1999; Gibson, 2000; Moore et al., 2003). Interestingly, HXK also seems to participate in the sugar regulation of *Nia1* nitrate reductase gene expression in *Arabidopsis* (Jang et al., 1997; Sheen et al., 1999), suggesting that it may be an important regulatory element for the crosstalk between N and C metabolisms. One argument in support of the signaling role of HXK is that the sugar analogs 2-deoxyglucose (2-DOG) and mannose are able to mimic the action of glucose on the regulation of gene expression, whereas 3-O-methylglucose (3-OMG) is not (Jang and Sheen, 1994). Both 2-DOG and mannose can be recognized and phosphorylated by HXK but are metabolized poorly later during glycolysis, whereas 3-OMG can be taken up but not metabolized at all. These observations suggest that HXK is required and sufficient to generate the sugar signal, independent of the role of its substrate or product as a carbon source for the cell.

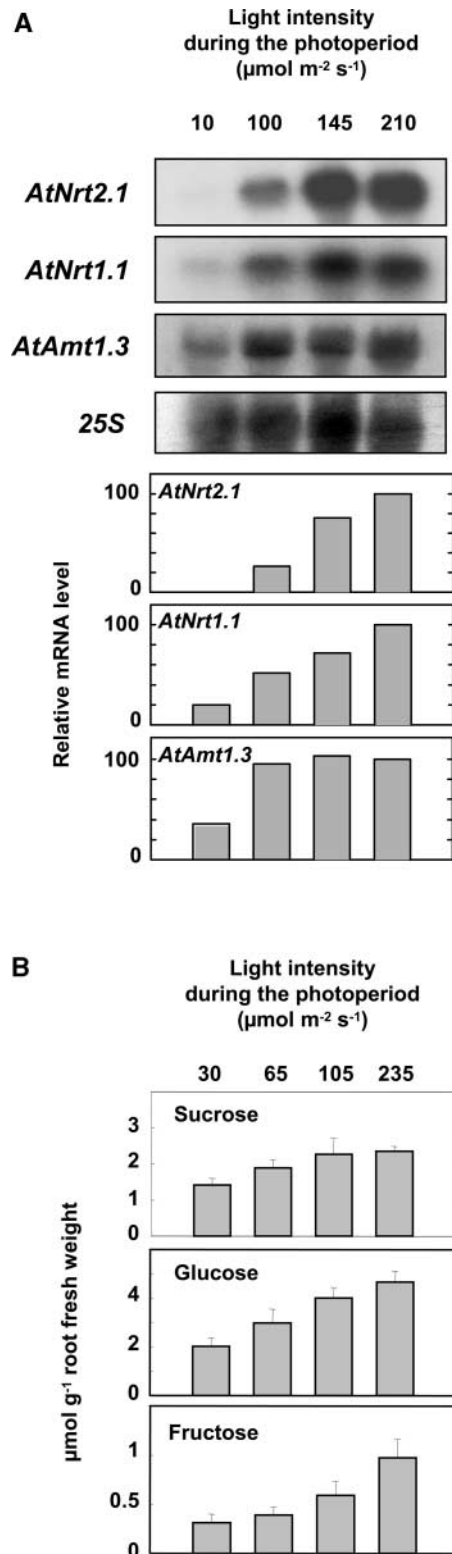


Figure 6. Effect of Light Intensity on Ion Transporter Gene Expression and Sugar Accumulation in the Roots.

(A) RNA gel blot analysis of the expression of *AtNrt2.1*, *AtNrt1.1*, and *AtAmt1.3* genes in the roots. Six-week-old plants were grown on a com-

Although the data obtained with these sugar analogs should be considered with caution with regard to their exact fate in plant cells (Klein and Stitt, 1998), we investigated their effect on the regulation of *AtNrt2.1* expression, together with that of glucosamine, an inhibitor of HXK. If the signaling function of HXK is involved in the induction of *AtNrt2.1* expression, both 2-DOG and mannose should stimulate this expression. However, the reverse was observed, and 2-DOG or mannose supply to the roots for 4 h resulted in a dramatic decrease in *AtNrt2.1* transcript accumulation (Figure 10A). Glucosamine also reduced *AtNrt2.1* transcript level significantly, even when sucrose was supplied simultaneously (Figure 10A). These results indicate that HXK activity is required, but not sufficient per se, to maintain the expression of *AtNrt2.1*. Similar results were found with *AtNrt1.1*, *AtAmt1.2*, *AtAmt1.3*, and *AtHst1* (data not shown), all of which display marked diurnal changes in expression and strong induction by sucrose (Figure 3). However, expression of *AtAmt1.1*, which was affected only slightly by the light/dark transition and sucrose supply (Figure 3), was much less sensitive to 2-DOG, mannose, and glucosamine (data not shown). Supply of 3-OMG had no effect on *AtNrt2.1* expression (Figure 10A) or on that of all other transporter genes (data not shown).

To obtain information on the action of the sugar analogs and glucosamine at the functional level, both root $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ influxes were measured during the same experiments used in the gene expression studies. The activity of the HATS for NO_3^- was affected by sugar or sugar analog supply in the same way as *AtNrt2.1* transcript level (cf. Figures 10A and 10B). The HATS for NH_4^+ also was inhibited by 2-DOG, mannose, and glucosamine, but to a lesser extent than the HATS for NO_3^- (data not shown). The activity of the low-affinity transport system for NO_3^- remained remarkably constant between the treatments (Figure 10B). This finding, together with the limited action of 2-DOG and mannose on *AtAmt1.1* expression, shows that the negative effect of the sugar analogs on the expression of some of the transporter genes likely was not the result of toxicity.

Sugar Regulation of *AtNrt2.1* Expression Is Not Affected in *rsr1-1*, *sun6*, and *gin1-1* Sugar-Sensing Mutants

The regulation of *AtNrt2.1* expression was investigated in three of the known Arabidopsis sugar-sensing mutants, *rsr1-1* (Martin et al., 1997), *sun6* (Van Oosten et al., 1997), and *gin1-1*

plete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle at normal light intensity ($210 \mu\text{mol m}^{-2} \text{s}^{-1}$). On the day of the experiment, all plants were illuminated at the usual beginning of the light period (09 h). One group of plants was illuminated at the normal light intensity, and three other groups were shaded to decrease the light intensity at the canopy height to 145, 100, and $10 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. The plants were harvested for RNA extraction at the end of the light period (17 h) after 8 h of treatment. Transcript accumulation was quantified after normalization using a 25S probe.

(B) Sucrose, glucose, and fructose concentrations in the roots. The experimental conditions are the same as those described for **(A)**. The data shown are means of four replicates \pm SE.

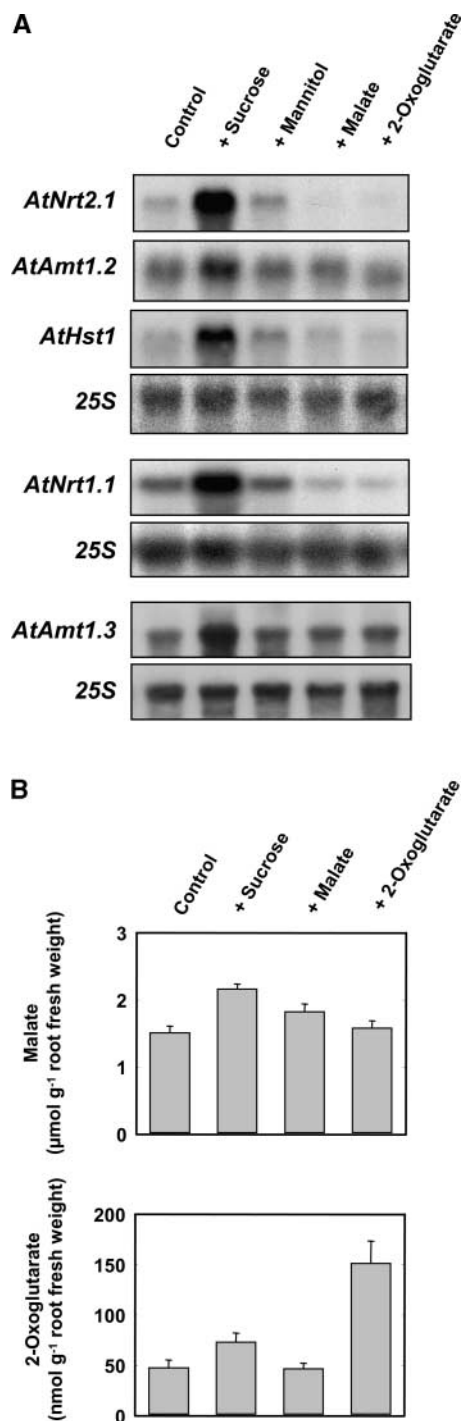


Figure 7. Effect of Sucrose, Malate, and 2-Oxoglutarate on Ion Transporter Gene Expression in the Roots.

(A) RNA gel blot analysis of the expression of *AtNrt2.1*, *AtNrt1.1*, *AtAmt1.2*, *AtAmt1.3*, and *AtHst1* genes in the roots in response to sucrose, mannitol, malate, or 2-oxoglutarate supply during the night. Six-week-old plants were grown on a complete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle. The various metabolites were supplied in the nutrient solution (15 mM final concentration for sucrose and mannitol, 10 mM for malate and 2-oxoglu-

(Zhou et al., 1998). The mutant *rsr1-1* displayed a strongly reduced induction of the class-I patatin promoter by sucrose. This promoter also was inducible by 3-OMG, suggesting that the origin of the signal transduction pathway is located upstream of HXK. In the *sun6* mutant, the activity of the plastocyanin promoter was not repressed by 2-DOG, and sensitivity to high glucose concentration in the early stages of shoot development was reduced. In *gin1-1*, cotyledon development after germination also was insensitive to glucose inhibition, and glucose repression of photosynthesis genes was suppressed. Both *sun6* and *gin1-1* mutants have been proposed to be affected in the HXK-related signal transduction pathway (Van Oosten et al., 1997; Zhou et al., 1998). In all three mutants, neither the diurnal changes nor the sugar inducibility of *AtNrt2.1* expression was modified significantly compared with that of the wild-type or parent lines (Figure 11). On the one hand, the unaltered expression of *AtNrt2.1* in *rsr1-1* is consistent with the absence of effect of 3-OMG (Figure 10) and indicates that signaling is not related to hexose transport. On the other hand, the observations that *AtNrt2.1* is regulated normally in *sun6* and *gin1-1* and that 2-DOG and mannose do not mimic the action of glucose or sucrose are in agreement with the conclusion that specific signaling by HXK is not involved in the control of *AtNrt2.1* expression.

Underexpression of HXK Abolishes the Induction of *AtNrt2.1* by Sugars

The observation that the induction of *AtNrt2.1* expression by sucrose is independent of HXK-related signaling but is suppressed when HXK is inhibited by glucosamine (Figure 10A) suggests that the catalytic function of the enzyme is crucial for the sugar regulation of *AtNrt2.1*. This notion was verified using transgenic plants underexpressing or overexpressing *AtHXK1* or expressing yeast *YHXK2* (Jang et al., 1997). When expressed in Arabidopsis, *YHXK2* is able to fulfill the catalytic function, but not the signaling function, of the plant HXK. Thus, in plants expressing *YHXK2*, total HXK activity is increased compared with that in the wild type, but signaling by the plant HXK is altered as a result of dominant-negative interaction (Jang et al., 1997). When investigated during the dark period, the usual stimulation of *AtNrt2.1* expression by sugars was found in all plant lines except antisense *AtHXK1* transformants (Figure 12), in which *AtNrt2.1* transcript accumulation was higher than in the wild type in the absence of light and no more stimulated by either sucrose or glucose. Thus, altering HXK-related signaling with *YHXK2* expression had no effect on *AtNrt2.1* transcript accu-

tarate, pH adjusted to 5.8) with antibiotics (50 mg/L penicillin and 25 mg/L chloramphenicol) at the beginning of the dark period (17 h), and the plants were harvested 4 h later. Antibiotics alone were supplied to control plants. RNA sample loading was quantified using a 25S probe.

(B) Malate and 2-oxoglutarate concentrations in the roots after 4 h of exogenous supply of sucrose, malate, or 2-oxoglutarate during the night period. The plants were from the same experiment described for **(A)**. The results shown are means of four replicates \pm SE.

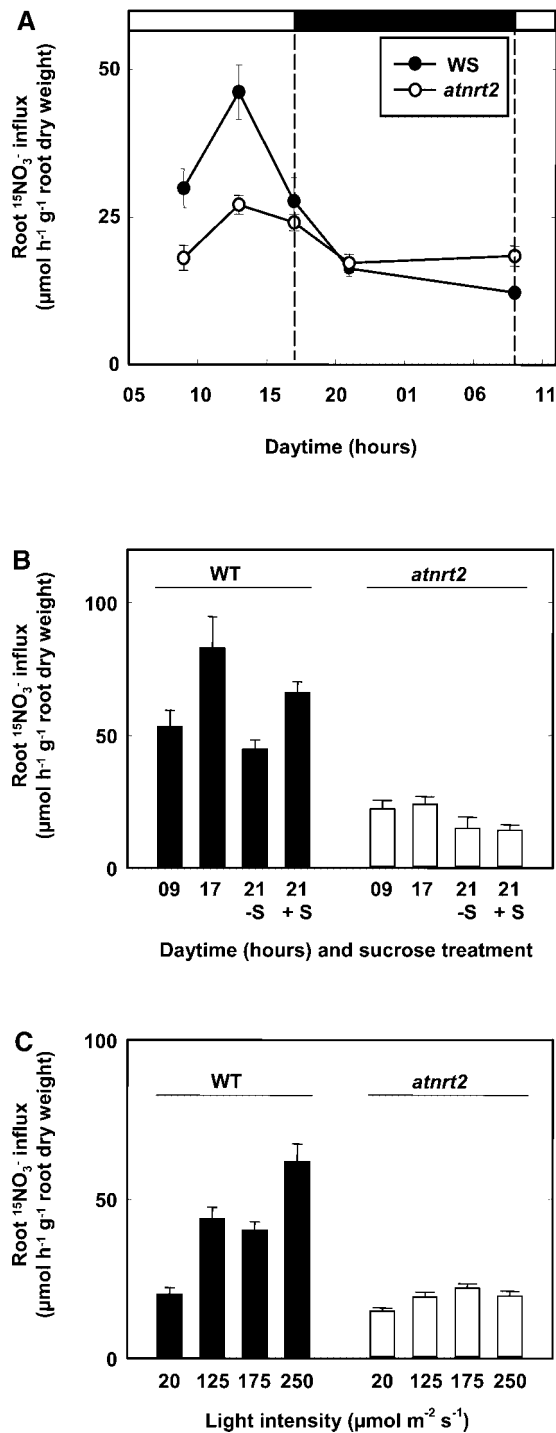


Figure 8. Effect of Light and Sucrose on NO₃⁻ Uptake in Wild-Type and *atnrt2* Mutant Roots.

(A) Diurnal changes of root ¹⁵NO₃⁻ influx in wild-type Wassilewskija (WS) and *atnrt2* plants. The plants were grown on a complete nutrient solution containing 1 mM NH₄NO₃ as an N source until the age of 5 weeks under an 8-h-day/16-h-night cycle. One week before the experiments, the plants were transferred to a nutrient solution containing 1 mM NO₃⁻ as the sole N source. Root ¹⁵NO₃⁻ influx was measured at

200 μM external ¹⁵NO₃⁻ concentration. The results shown are means of 12 replicates ± SE.

DISCUSSION

Induction by Sugars Is a Main Feature of the Regulation of Ion Transporter Gene Expression in the Root

Compared with our previous investigations with NO₃⁻ or NH₄⁺ transporter genes (Gazzarrini et al., 1999; Lejay et al., 1999), here we expanded our study of the regulation of root ion transport by light and sugars to a larger number of genes that encode transporters and channels for a variety of other important nutrients (SO₄²⁻, K⁺, Pi, Fe, and other metal ions). Expression studies with this set of genes led to two main conclusions. First, a significant proportion of them displayed diurnal changes in expression and were inducible by sucrose. This finding suggests that the control exerted by light and sugars is a key regulatory process for transporter or channel proteins in the roots. This conclusion agrees with observations made in shoots that light regulates two H⁺-ATPase genes (of three investigated) in tomato (Mito et al., 1996) and all four K⁺ channels identified in the pulvinus of the legume *Samanea saman* (Moshelion et al., 2002). Second, a strong correlation was found between the responses of transcript accumulation to illumination of the plant on the one hand and to sucrose supply to the roots on the other hand (Figure 3). This finding suggests that the diurnal changes in gene expression result from the decreased downward transport of sucrose to the roots at night (Riems et al., 1994) and the concomitant decrease in the root concentrations of soluble sugars (Figure 4) (Kerr et al., 1985; Ruffy et al., 1989). The effect of light seen in this study probably relates to a regulation exerted by photosynthates. This is in agreement with earlier studies with CO₂-free air, showing that the diurnal changes of root NO₃⁻ uptake were caused by carbon assimilation and not by the action of light per se (Delhon et al., 1996).

As stated above, the most pronounced effects of the day/night transition and of sugar supply were seen for NO₃⁻,

200 μM external ¹⁵NO₃⁻ concentration. The results shown are means of 12 replicates ± SE.

(B) Diurnal changes and sucrose stimulation of root ¹⁵NO₃⁻ influx in wild-type (WT) and *atnrt2* plants. Culture of the plants and assay of ¹⁵NO₃⁻ influx were performed as described for **(A)**. Root ¹⁵NO₃⁻ influx was measured at the beginning (09 h) and at the end (17 h) of the light period and 4 h into the dark period (21 h) in plants supplied with (+S) or without (-S) 1% sucrose. The results shown are means of 12 replicates ± SE.

(C) Effect of light intensity on root ¹⁵NO₃⁻ influx in wild-type and *atnrt2* plants. Culture of the plants and assay of ¹⁵NO₃⁻ influx were performed as described for **(A)**. Root ¹⁵NO₃⁻ influx was measured at the end of the light period. During the light period preceding the influx measurements, the plants were either illuminated at the normal light intensity (250 μmol·m⁻²·s⁻¹) or shaded to decrease the light intensity to 175, 125, or 20 μmol·m⁻²·s⁻¹, as described for Figure 6. The results shown are means of 12 replicates ± SE.

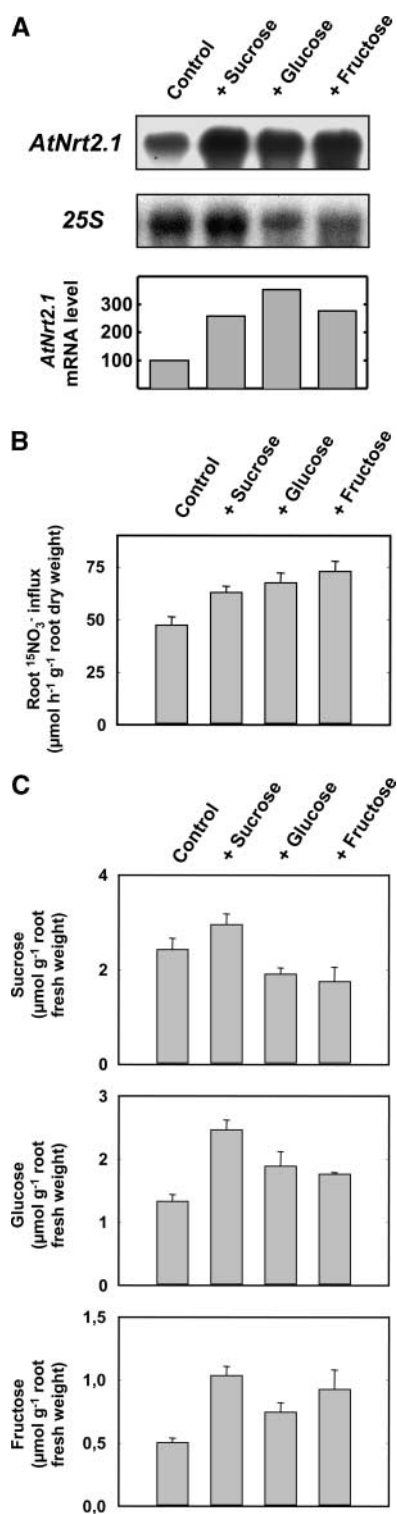


Figure 9. Effect of Various Sugars on NO₃⁻ Uptake and Expression of *AtNrt2.1* in the Roots.

(A) RNA gel blot analysis of the expression of *AtNrt2.1* in the roots in response to sucrose, glucose, or fructose supply during the night. Six-week-old plants were grown on a complete nutrient solution containing

NH₄⁺, and SO₄²⁻ transporters (*AtNrt1.1*, *AtNrt2.1*, *AtAmt1.2*, *AtAmt1.3*, and *AtHst1*) (Figure 3). This finding prompted us to investigate the hypothesis that carboxylic acids, which are the carbon skeletons for amino acid synthesis, might be the actual signaling compounds for the regulation of NO₃⁻, NH₄⁺, and SO₄²⁻ transporter gene expression. This hypothesis is rejected on the basis that neither malate nor 2-oxoglutarate was able to induce the root ion transporters for NO₃⁻, NH₄⁺, and SO₄²⁻ (Figure 6). This is surprising because 2-oxoglutarate is a very popular candidate as a regulatory molecule in fungi (Crawford and Arst, 1993) and in plants, in which it is believed to stimulate NR gene expression (Ferrario-Mery et al., 2001). In addition, malate has been shown to stimulate root NO₃⁻ uptake (Touraine et al., 1992). However, our results showing no stimulation of ion transporter gene expression by malate and 2-oxoglutarate agree with the recent report that neither compound stimulates NR transcript accumulation in tobacco (Müller et al., 2001). Also, the positive effect of malate on NO₃⁻ uptake was not attributed initially to transcriptional control but rather to the occurrence of a NO₃⁻/HCO₃⁻ antiport, stimulated by malate decarboxylation in the roots (Touraine et al., 1992).

Fast decarboxylation of malate supplied to the roots was observed previously (Touraine et al., 1992) and may explain why this compound did not accumulate in these organs after addition in the nutrient solution (Figure 7). Finally, induction by sugars also was found for genes that encode transporters for ions that are not incorporated in amino acids (Pi) or not even metabolized (K⁺). This finding suggests a more general control, not restricted to amino acid synthesis, that is in agreement with the fact that the light/dark transition affects the root uptake of many ions (Clément et al., 1978; Smith and Cheema, 1985; Hatch et al., 1986; Le Bot and Kirkby, 1992; Delhon et al., 1995a). In shoots, light and/or sugars also regulate the expression of K⁺ channel genes (Deeken et al., 2000; Ache et al., 2001; Moshelion et al., 2002) in addition to NH₄⁺ transporter genes (von Wirén et al., 2000b).

Mutation of *AtNrt2.1* Results in the Loss of Coordination between High-Affinity NO₃⁻ Uptake and Photosynthesis

Despite the variety of ion transporter and channel genes already shown to be regulated by light and photosynthesis in

1 mM NO₃⁻ as an N source under an 8-h-day/16-h-night cycle. The various metabolites were supplied in the nutrient solution (10 mM final concentration) with antibiotics (50 mg/L penicillin and 25 mg/L chloramphenicol) at the beginning of the dark period (17 h), and the plants were harvested 4 h later. Antibiotics alone were supplied to control plants. Transcript accumulation was quantified after normalization using a 25S probe.

(B) Effect of sucrose, glucose, or fructose supply on root ¹⁵NO₃⁻ influx during the night. The plants are from the same experiment described for **(A)**. Root ¹⁵NO₃⁻ influx was measured at 200 μM external ¹⁵NO₃⁻ concentration. The results shown are means of 12 replicates ± SE.

(C) Sucrose, glucose, and fructose concentrations in the roots of plants supplied with these metabolites for 4 h in the dark. The plants are from the same experiment described for **(A)**. The results shown are means of four replicates ± SE.

both roots and shoots (Gazzarrini et al., 1999; Lejay et al., 1999; Deeken et al., 2000; Ono et al., 2000; von Wirén et al., 2000b; Ache et al., 2001; Matt et al., 2001; Moshelion et al., 2002), functional evidence that these genes are responsible for the ef-

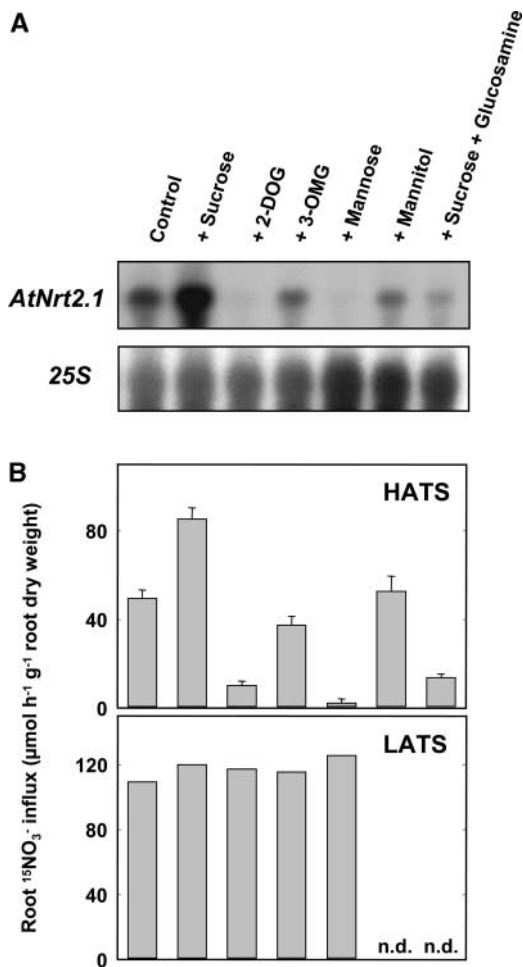


Figure 10. Effect of Sugar Analogs and Glucosamine on NO_3^- Uptake and *AtNrt2.1* Gene Expression in the Roots.

(A) RNA gel blot analysis of *AtNrt2.1* expression in roots of plants supplied for 4 h with sucrose (30 mM), 2-DOG (0.5 mM), 3-OMG (10 mM), mannose (10 mM), mannitol (30 mM), and sucrose (30 mM) plus glucosamine (20 mM). Six-week-old plants were grown on a complete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle. The various metabolites were supplied at the beginning of the dark period (17 h), and the plants were harvested 4 h later. Antibiotics were supplied to all plants (including control plants) during the treatments. RNA sample loading was quantified using a 25S probe.

(B) Effect of sucrose, 2-DOG, 3-OMG, mannose, mannitol, and sucrose plus glucosamine on root $^{15}\text{NO}_3^-$ influx mediated by both HATS and the low-affinity transport system (LATS). The experimental conditions were the same as those described for **(A)**. HATS activity was determined by measuring root $^{15}\text{NO}_3^-$ influx at 200 μM external $^{15}\text{NO}_3^-$ concentration. LATS activity was calculated as the difference between root $^{15}\text{NO}_3^-$ influxes measured at 5 mM and 200 μM . Root $^{15}\text{NO}_3^-$ influx was measured on 12 replicates, and results for HATS are presented \pm SE. n.d., not determined.

fect of light and/or sugars on ion transport itself has not been provided to date. Our results obtained with the *atnrt2* mutant (Figure 8), however, indicate that the mutation of a transporter gene results in the loss of regulation of root ion uptake by photosynthesis. Both *AtNrt2.1* and *AtNrt2.2* are mutated in *atnrt2* plants (Filleur et al., 2001). However, the expression of *AtNrt2.2* has not been observed under our conditions, even using reverse transcriptase-mediated PCR (Orsel et al., 2002). This finding strongly suggests that the phenotype of the mutant is related to the deletion of *AtNrt2.1*, which then plays a central role in the coordination of root NO_3^- uptake and photosynthesis.

These data suggest that this coordination is the result of the regulation of *AtNrt2.1* transcript synthesis and/or stability by sugars, which may modulate the density of AtNRT2.1 proteins in membranes according to the C status of the plant. Nevertheless, we cannot exclude the hypothesis that regulation of AtNRT2.1 expression at levels other than the mRNA also may be involved. For instance, the accumulation of *Nia* transcripts (which encode NR) was found to be strongly stimulated by sucrose supply in *Arabidopsis* and *Nicotiana plumbaginifolia* leaves (Cheng et al., 1992; Vincenz et al., 1993), but it is likely that the regulation of NR at the post-translational level also is crucial in linking NO_3^- reduction with photosynthesis (Kaiser and Huber, 1994). Although nothing is known about the regulation of AtNRT2.1 at the protein level, the post-transcriptional regulation of the NRT2 transporter has been suspected in *N. plumbaginifolia*, in which constitutive expression of *NpNrt2.1*, resulting in a constant transcript level in roots, did not prevent the repression of high-affinity NO_3^- uptake by NH_4^+ supply to the plant (Fraisier et al., 2000). Nonetheless, the *atnrt2* mutant provides a unique example of a genotype in which high-affinity root NO_3^- uptake is uncoupled from shoot photosynthesis, indicating that the coordination of both functions relies on specific transporter genes and is not simply the result of a general modulation of the energization of secondary active ion transport in the roots. Because the *atnrt2* mutant has been shown to be affected in other major regulatory pathways that govern high-affinity NO_3^- uptake, such as induction by NO_3^- and repression by high N status or N metabolites (Cerezo et al., 2001), the *AtNrt2.1* gene is likely to be a key transporter gene responsible for the integration of high-affinity root NO_3^- uptake with both N and C metabolism at the whole-plant level.

The Mechanisms Involved in the Sugar Regulation of *AtNrt2.1*: A Signal Dependent on Glycolytic Flux?

The mechanisms of the sugar regulation of gene expression have been investigated extensively using biochemical, pharmacological, and genetic approaches (for recent reviews, see Sheen et al., 1999; Gibson, 2000; Smeekens, 2000; Coruzzi and Zhou, 2001; Rolland et al., 2002). At least three different signaling pathways have been documented in plants: a regulation involving specific signaling by sucrose (Chiou and Bush, 1998), a regulation involving the transport step of hexoses as the origin of the signal (Martin et al., 1997; Lalonde et al., 1999), and a regulation involving HXK as a sugar sensor (Jang and Sheen, 1994; Jang et al., 1997; Moore et al., 2003). In spite of

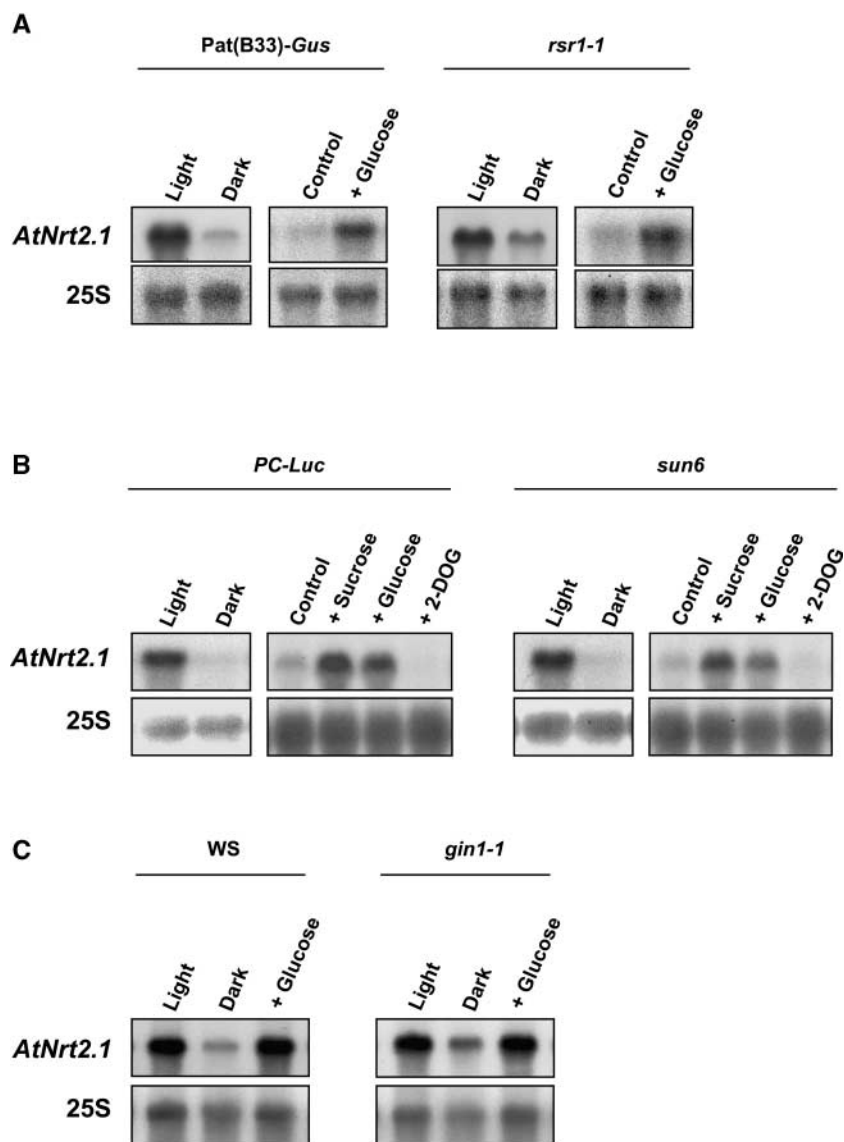


Figure 11. Diurnal Changes and Sugar Induction of *AtNrt2.1* Gene Expression in Sugar-Sensing Mutants.

(A) RNA gel blot analysis of *AtNrt2.1* expression in the roots of *rsr1-1* mutant and Pat(B33)-Gus control plants. Six-week-old plants were grown on a complete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle. Plants were harvested at the end of the light period (Light) and 4 h into the dark period (Dark) or, in another experiment, 4 h into the dark period with (+ Glucose) or without (Control) exogenous glucose at 15 mM after the end of the light period. Antibiotics were supplied to all plants (including control plants) during the treatments. RNA sample loading was quantified using a 25S probe.

(B) RNA gel blot analysis of *AtNrt2.1* expression in the roots of *sun6* mutant and PC-Luc control plants. The growth and treatment conditions were the same as those described for **(A)** except that the glucose concentration was 60 mM and the exogenous metabolite treatment also was performed with sucrose (60 mM) and 2-DOG (2 mM). RNA sample loading was quantified using a 25S probe.

(C) RNA gel blot analysis of *AtNrt2.1* expression in the roots of *gin1-1* mutant and wild-type Wassilewskija (WS) plants. The growth and treatment conditions were the same as those described for **(A)** except that the dark treatment also served as the control for glucose supply. RNA sample loading was quantified using a 25S probe.

the numerous reports dealing with these three hypotheses, none included ion channel or transporter genes in the target genes investigated. The only available evidence concerning mineral nutrition is the observation that *Nia1* gene expression is stimulated in sense-HXK transformants of *Arabidopsis*, sug-

gesting a role for HXK in the regulation of NR expression (Jang et al., 1997).

Our results indicate that none of the three signaling pathways described above is involved in the sugar regulation of *AtNrt2.1* expression. The induction of *AtNrt2.1* by sugars also

was observed after both glucose and fructose treatment, without any increase in sucrose concentration in the roots (Figure 9). This finding precludes a predominant role for sucrose-specific signaling. Two observations support the hypothesis that signaling by hexose transport is not responsible for the regulation of *AtNrt2.1* expression. First, the nonmetabolizable glucose analog 3-OMG had no effect on *AtNrt2.1* transcript accumulation (Figure 10). Second, the *rsr1-1* mutant, which was shown to be altered in hexose transport-mediated signaling for the regulation of the class-I patatin promoter (Martin et al., 1997), displayed no modification of either the diurnal or the sugar-mediated regulation of *AtNrt2.1* expression in roots (Figure 11). Similar arguments are not in favor of a central role of HXK as the sugar sensor involved in the signaling of the sugar regulation of *AtNrt2.1* expression. The glucose analogs 2-DOG and mannose did not mimic the effect of sugars on *AtNrt2.1* transcript accumulation (Figure 10), and both *sun6* and *gin1-1*, which are altered in HXK-related signaling (Van Oosten et al., 1997, Zhou et al., 1998), were found to have unaltered *AtNrt2.1* expression patterns in response to both the light/dark transition and sucrose or glucose supply (Figure 11). Finally, the strongest argument to exclude a role for the signaling function of HXK in the regulation of *AtNrt2.1* transcript accumulation is the observation that the expression of the yeast *YHXK2* in transgenic plants did not alter the sugar induction of *AtNrt2.1* in the roots (Figure 12), despite the profound modifications of HXK-

related signaling resulting from this genetic transformation (Jang et al., 1997).

The apparent upregulation of *AtNrt2.1* expression in antisense *AtHXK1* plants not supplied with sugars is intriguing (cf. control anti-*AtHXK1* and wild-type plants in Figure 12), but it does not indicate a repressive action of HXK on *AtNrt2.1* expression, because this upregulation was not found after a short-term supply of glucosamine (Figure 10) or in sense *YHXK2* transformants (Figure 12). One hypothesis is that the upregulation of *AtNrt2.1* in antisense *AtHXK1* plants may result from the relief of negative feedback exerted by N metabolites (Lejay et al., 1999). Indeed, if NO_3^- uptake is not stimulated by sugars in HXK underexpressors, as expected from the lack of induction of *AtNrt2.1* by sucrose and glucose (Figure 12), the plants may suffer from N limitation during periods of active photosynthesis. This upregulation of *AtNrt2.1* is not found when HXK activity is inhibited by glucosamine, probably because the duration of glucosamine treatment (4 h) is too short to trigger upregulation by N limitation. Alternatively, it cannot be excluded that another hexose kinase than *AtHXK1* is responsible for the induction of *AtNrt2.1*. If this holds true, upregulation of *AtNrt2.1* in anti-*AtHXK1* transformants may result from the increased activity of this unknown hexose kinase as a result of either compensatory upregulation or greater sugar availability in response to the reduced activities of both *AtHXK1* and *AtHXK2* in antisense *AtHXK1* plants (Jang et al., 1997).

Although our data do not allow the precise identification of the signaling pathway involved in the sugar regulation of *AtNrt2.1* expression, two lines of evidence suggest that it is strongly dependent on the carbon flux in glycolysis. First, the sugar induction of *AtNrt2.1* expression was abolished in antisense *AtHXK1* transformants (Figure 12) and in wild-type plants supplied with the HXK inhibitor glucosamine (Figure 10). This finding indicates that although HXK signaling function is not involved in the regulation of *AtNrt2.1*, its catalytic activity is required for the sugar induction of this gene. Second, the strong negative effects of both 2-DOG and mannose (Figure 10) are consistent with a role of the products of HXK activity in controlling *AtNrt2.1* expression. An explanation for this observation might be that 2-DOG and mannose compete with glucose, the natural substrate of HXK, and lead to a reduction of the downstream glycolytic flux. Indeed, treatment of *Chenopodium rubrum* cells with either 2-DOG or mannose results in the decreased accumulation of phosphorylated glycolytic intermediates and of ATP (Klein and Stitt, 1998).

Collectively, these observations indicate that a transduction pathway related to sugar metabolism downstream of the reaction catalyzed by HXK is responsible for the induction of *AtNrt2.1* expression by photosynthates. A similar hypothesis has been proposed to explain the sugar stimulation of the insulin gene promoter in mammals (German, 1993). Our conclusion also has to be related to the previous proposal of a glycolysis-dependent pathway in plants (Sheen et al., 1999; Rolland et al., 2002), based on the observation that HXK activity, but not its signaling function, is required for the sugar induction of two PR genes in *Arabidopsis* (Xiao et al., 2000). Interestingly, *AtNrt1.1*, *AtAmt1.3*, and *AtHst1* were found to respond to light, sugars, carboxylic acids, glucosamine, and

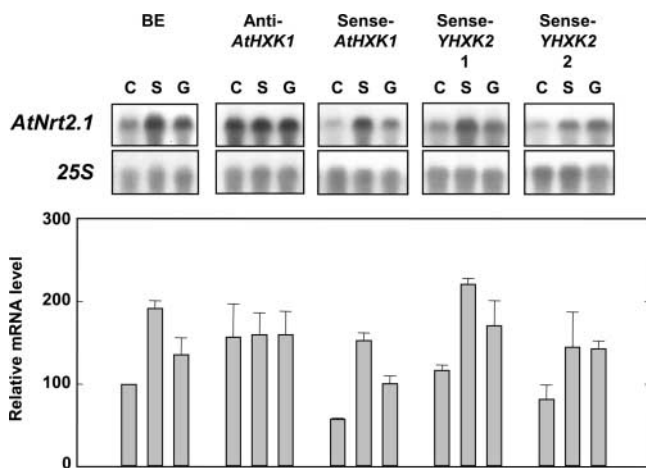


Figure 12. Sugar Induction of *AtNrt2.1* Gene Expression in Transgenic Lines Underexpressing or Overexpressing HXK.

RNA gel blot analysis of *AtNrt2.1* expression in the roots of wild-type (ecotype Beinsheim; BE), antisense, and sense *AtHXK1* lines and two independent lines overexpressing yeast *YHXK2*. Six-week-old plants were grown on a complete nutrient solution containing 1 mM NO_3^- as an N source under an 8-h-day/16-h-night cycle. Plants were harvested 4 h into the dark period and supplied after the end of the light period with 15 mM sucrose plus antibiotics (S), 15 mM glucose plus antibiotics (G), or antibiotics only for the controls (C). Transcript accumulation was quantified after normalization using a 25S probe, and the data shown are means from four replicates \pm SE (two different analyses from each of two independent experiments).

sugar analogs in the same way as *AtNrt2.1* (Figures 1 to 3 and 5 to 7). This finding suggests that the conclusions described above concerning the regulation of *AtNrt2.1* by sugars may hold true for several root ion transporters. Thus, our data support the hypothesis that the yet uncharacterized glycolysis-dependent pathway for sugar sensing in plants also may be involved in the control of root nutrient acquisition and then may have a more general significance than was suspected initially.

METHODS

Culture of the Plants

Plants of the various genotypes of *Arabidopsis thaliana* used in this study were grown hydroponically under nonsterile conditions as described by Lejay et al. (1999). Briefly, the seeds were germinated directly on top of modified Eppendorf tubes filled with prewetted sand. The tubes then were positioned on floating rafts transferred to tap water in a growth chamber under the following environmental conditions: light/dark cycle of 8 h/16 h, light intensity of 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, temperature of 22/20°C, and RH of 70%. After 1 week, the tap water was replaced with a complete nutrient solution. Most of the experiments were performed on plants grown on 1 mM NO_3^- as an N source, except those involving the assay of root NH_4^+ uptake or the investigation of the *atnrt2* mutant, for which the N source supplied to the plants was 1 mM NH_4NO_3 . The other nutrients were added as described by Lejay et al. (1999). The plants were allowed to grow for 5 additional weeks before the experiments. Nutrient solutions were renewed weekly and on the day before the experiments. pH was adjusted to 5.8. All experiments were repeated two or three times, and typical results are shown.

Supply of Carbon Metabolites, Sugar Analogs, or Inhibitor

Shading of the plants was performed by installing a nylon mesh screen between the light source and the culture canopy. Decreasing levels of light intensity (quantified using a light probe) were obtained by increasing the number of layers of nylon mesh.

The treatments involving the supply of sugars or carboxylic acids into the nutrient solution were performed according to the procedure described previously (Lejay et al., 1999). Immediately after light was switched off, the plants were transferred to fresh nutrient solution, pH 5.8, supplemented with the various compounds investigated at the concentrations indicated in the figures. For all experiments involving the supply of carbon metabolites, 50 mg/L penicillin and 25 mg/L chloramphenicol were added to the nutrient solution. Control plants were transferred to fresh nutrient solution supplemented with the antibiotics only. After harvest, all plant tissues were weighted and frozen at -80°C , except the samples for the ^{15}N assay.

Root Influxes of $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$

Root influxes of $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ were assayed as described by Delhon et al. (1995a) and Gazzarrini et al. (1999), respectively. The plants were transferred sequentially to 0.1 mM CaSO_4 for 1 min and to a complete nutrient solution, pH 5.8, containing either $^{15}\text{NO}_3^-$ or $^{15}\text{NH}_4^+$ (99% atom excess ^{15}N) for 5 min at the concentrations indicated in the figures. At the end of the ^{15}N labeling procedure, roots were washed for 1 min in 0.1 mM CaSO_4 and separated from shoots. The organs were dried at 70°C for 48 h, weighed, crushed in a hammer mill, and analyzed for total ^{15}N content using a continuous-flow isotope ratio mass spectrometer

coupled with an C/N elemental analyzer (model ANCA-MS; PDZ Europa, Crewe, UK), as described by Clarkson et al. (1996).

RNA Gel Blot Analysis

RNA extraction was performed as described previously (Lobreaux et al., 1992). Total RNA (10 μg) was separated by electrophoresis on 3-(*N*-morpholino)-propanesulfonic acid-formaldehyde agarose gels and blotted onto nylon membranes (Hybond N^+ ; Amersham). Membranes were prehybridized for 2 h at 60°C in 0.5 M NaH_2PO_4 , 1% BSA, 7% SDS, pH 7.2, with H_3PO_4 . Hybridization was performed overnight at 60°C after addition of the randomly primed ^{32}P -labeled cDNA probe in the prehybridization buffer. Membranes were washed twice at room temperature for 2 min and twice at 60°C for 15 min with $0.5\times$ SSC ($1\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS. The DNA probes used in this study correspond to either the full-length cDNA (*AtNrt2.1*, *AtNrt1.1*, *AtAmt1.1*, *AtAmt1.2*, *AtAmt1.3*, *AtIrt1*, *AtNramp1*, *AtSkor*, *AtAkt1*, and *AtHst1*) or a cDNA fragment (5' fragment of 2 kb for *AtKup2*, EST 200L10T7 for *AtPt1*, and EST 178H14XP for *AtPt2*). A 25S rRNA probe was used as a reference for quantification achieved using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Analysis of Metabolites

Extraction and analysis of metabolites was performed as described previously (Müller et al., 2001). Frozen root tissue was ground to a fine powder in a mortar precooled with liquid N_2 and extracted with ethanol, and the extracts were stored at -20°C . Malate, 2-oxoglutarate, and sugars were measured enzymatically (Stitt et al., 1989).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Alain Gojon, gojon@ensam.inra.fr.

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Regulation of Root Ion Transporters by Photosynthesis: Functional Importance and Relation with Hexokinase

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