# Identification of Sugar Signals Controlling the Nitrate Uptake by Rice Roots Using a Noninvasive Technique

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In order to evaluate the hypothesis that nitrate uptake is under positive control by sugars, a simple noninvasive model was used to measure the effects of nine modulating sugars on the net nitrate uptake in rice under constant low nitrate concentration. The results showed that the fastest and greatest acceleration in nitrate uptake were observed after sucrose was added, and this change reached a peak 1.5 h after treatment. In addition, we found that 1 mM sucrose could affect the nitrate uptake in rice roots for 8.5 h. The three most positive effective sugars and one negative effective sugar were selected for further analyzing their effect on the expression of nitrate transporter gene *OsNrt2.1*. The result of RT-PCR showed that the expression of *OsNrt2.1* was upregulated by sucrose, glucose and galactose. Among the three positive effective sugars tested, sucrose was found to have significant and continuous enhanced stimulation on *OsNrt2.1* gene expression within 4 h, which indicated that sucrose could be as a specific signal to regulate the net nitrate uptake.

*Key words:* Noninvasive Technique, Sugars, *OsNrt2.1* Gene

## Introduction

Nitrate is the most important source of mineral nitrogen for higher plants during their growth and development. The uptake and assimilation of nitrate by roots is known to be changed with its supply in a manner suggesting that the nitrogen status of plants is probably sensed and can feedback to regulate this uptaking process. Many investigations have already proved the existence of modulating mechanisms to answer the regulation of nitrate uptake according to the plant requirement (Crawford and Glass, 1998; Imsande and Touraine, 1994). There are two different modulating mechanisms about the adjustment of nitrate uptake. The first one is a positive feedback regulation. The acceleration of light and/or photosynthesis can increase the amount of sugars in shoots. Sugars have been shown to have several effects on the nitrogen reductive assimilation; therefore, the sugars moving to the roots in the phloem may act as a signal. The second mechanism is the regulation of the $NO_3^-$ uptake in relation to the nitrogen status of the plant and is thought probably to be mediated by feedback repression of $NO_3^-$ uptake systems by nitrogen metabolites that accumulate under conditions of nitrogen sufficiency (King *et al.*, 1993; Lee *et al.*, 1992). If plant growth speeds up, nitrogen arriving from roots will be used up faster; that results in the fall of the concentration of free amino acids and other nitrogen compounds. The flow of reduced nitrogen to the root system will also fall, and it can act as a signal to increase the nitrate uptake and transport rates (Muller *et al.*, 1996; Geßler *et al.*, 1998a, b).

Nitrate assimilation is regarded to be connected with the photosynthetic process in plants. There are three different hypotheses about the nature of the relationship between assimilation products and nitrate uptake. The first one is based on the energy supplement. Nitrate uptake is an energy-dependent process and therefore depends on a continual supply of sugar as respiratory substrate. It is a very old-fashioned hypothesis because energy supply is not a rate-determining factor in biological processes except under extreme conditions (Cram, 1976, 1983). The second one is based on the control of the internal ion contents, such as $K^+$ (Siddiqi and Glass 1982, 1986), $H^+$ (Felle, 2001),

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$Ca^{2+}$ (Sanders *et al.*, 2002), $Cl^-$ (Cram, 1983), and $NO_3^-$ (Scaife, 1989; Buysse *et al.*, 1996; Walch-Liu *et al.*, 2005). This hypothesis is set up depending on the negative correlation between the nitrate uptake rate and the plant internal ion concentration. The third hypothesis is based on the feedback regulation by special molecules, such as sugars (Rufty *et al.*, 1989; Delhon *et al.*, 1996; Coruzzi and Bush, 2001).

At gene expression level, the modulation of nitrate uptake is probably put to practice by genes regulation encoding high-affinity $NO_3^-$ transporters when plants are fed with sugars (Lejay *et al.*, 2003), suggesting that sugars may act as feedback regulators of $NO_3^-$ uptake and assimilation. It is also supposed that the *AtNRT2.1* gene, which encodes high-affinity $NO_3^-$ transporters in *Arabidopsis*, is responsive to systemic shoot-derived signals relevant to the N status of the whole plant (Gansel *et al.*, 2001).

Sugars can probably regulate the pathways responsive to changes in the photosynthetic activity in shoots. These pathways refer to a wide range of nutrient uptake genes including the dual affinity $NO_3^-$ transporter, $NH_4^+$ transporters, high-affinity sulfate, phosphate and potassium transporters (Lejay *et al.*, 2003). It means that sugar-mediated diurnal regulation is not a specific modulation for $NO_3^-$ influx.

The aim of the present work was to test the hypotheses of the nitrate uptake regulation. Our general hypothesis was that the sugar signals are related to photosynthesis and potential growth and constitute a positive feed-forward signal. If the signal system really exists, the signal-regulating effect should be very fast. The signal molecule could affect the nitrate uptake directly, and the lag time would be short or even no lag phase exists. Some sugars might affect the nitrate uptake by interconverting to others; it is an indirect effect and the obvious lag time can be observed. Therefore, we wanted to find out: a) What are the sugar signal molecules regulating the nitrate uptake? b) How long do the signals alter the nitrate transport? What is the kinetics of response of nitrate uptake to the start of signaling?

## Material and Methods

### *Plant materials and growth conditions*

Seeds of rice (*Oryza sativa* cv. Shanyou63) were germinated on filter paper soaked with $H_2O$ for 48 h in the dark. Seedlings were transferred to hydroponic culture (Long Ashton solution) containing 10 mM nitrate, supplied as $Ca(NO_3)_2$ and $KNO_3$, in plastic containers that maintained the roots in the dark. The plants were allowed to grow in a controlled environment cabinet at 20 °C with a 16 h photoperiod (light on at 06:00 a.m. and light off at 10:00 p.m.), supplied with a photon flux density of 450–480 $\mu$mol $m^{-2}$ $s^{-1}$. Unless stated otherwise, all the feeding experiments began after 5–6 h in the light period.

### *Measurement of net nitrate uptake rates*

Two 21-day-old (18–25) plants of the same size were selected and transferred from the culture solution to a narrow plastic tray in water, the roots gently teased apart, and set up with the roots laid along a narrow plastic tray sloping at an angle of about 5°. A 10-fold dilution of Long Ashton solution was flowed from top to bottom of the root system at a flow rate of 0.3 ml $min^{-1}$. The plants were cultivated in the flowing system overnight before experiments.

The experiment was started at 11:00 or 12:00, and for the first 2 h, the two plants were in 1/10 Long Ashton solution. Then 1/10 Long Ashton solution containing a sugar (10 mM or 1 mM, depending on the experiment design) was pumped over one plant, while the solution pumped over the other plant remained unchanged. The solution pumped off was collected every 10 min in an automatic collector, and the concentration of nitrate in the samples was measured using a nitrate-selective microelectrode.

The $NO_3^-$ uptake rate was calculated as follows:

$$\text{nitrate uptake per min} = (C_{in} - C_{out}) \cdot (W_n - W_{n-1})/t,$$

where $C_{in}$ is the concentration of nitrate in nutrient solution, $C_{out}$ is the concentration of nitrate in nutrient solution after flowing over the roots, $(W_n - W_{n-1})$ is the difference between the weight of each test tube before and after collecting solution, and $t$ is the time collecting each sample.

The net nitrate influx was calculated according to

$$\varphi\text{net} = 60 \cdot (C_{in} - C_{out}) \cdot (W_n - W_{n-1})/t \cdot W_R,$$

where $W_R$ is the fresh weight of roots.

Full details of the set-up are given in Cram and Minchin (1994).

*Semi-quantitative RT-PCR*

Total RNA of the plants was extracted from roots using the Trizol reagent from Invitrogen. Reverse transcription-PCR (RT-PCR) was performed to investigate the gene expression with the access RT-PCR system from Promega. 100 ng cDNA were denatured at 70 °C for 5 min, and then 200 $\mu$mol dNTPs, 0.3 $\mu$M primers, 0.75 U *Taq* polymerase (Takara), and 10× buffer (Takara) were added in a final volume of 25 $\mu$l. The PCR program was as follows: 30 cycles at 94 °C for 5 min, at 94 °C for 45 s, at 57 °C for 45 s, and at 72 °C for 1 min, with a final extension at 72 °C for 10 min. 10 $\mu$l of the PCR product were loaded on 1% agarose gel to verify the amplification result and visualized under UV light. The sequences of the *OsNrt2.1* primers were as follows: forward primer, 5′-ccgcctctggaacatttgg-3′; reverse primer, 5′-tctcctgttcgctccactcg-3′. $\beta$-Actin of *Oryza sativa* was selected as the control gene, and the primer was shown as follows: forward primer, 5′-agatcatgtttgagaccttca-3′, reverse primer, 5′-gatatcaacatcacacttcat-3′.

## Results

*Effects of sugars on net nitrate uptake*

Nine different sugars were chosen, among which sucrose, glucose, and fructose are substrates in the phloem, and galactose, raffinose, mannitol, lactose, arabinose, and mannose are components of the cell wall. Lactose is a disaccharide only present in some special structure. These sugars are closely connected with photosynthesis or the key component of the cell wall. 10 mM of each sugar were chosen for saturating the transport system and at the same time avoiding significant osmotic effects. As examples of the most critical differences, the time courses of effects of different sugars were chosen to study the influence on the net nitrate uptake rate, and the data are shown in Figs. 1A–D. The net nitrate uptake rates went up rapidly after sucrose glucose, and galactose were added, and the lag time was distinctly different. The lag time of sucrose was within 10 min, and it was shortest compared with the other sugars. The duration of nitrate uptake accelerated by sucrose,

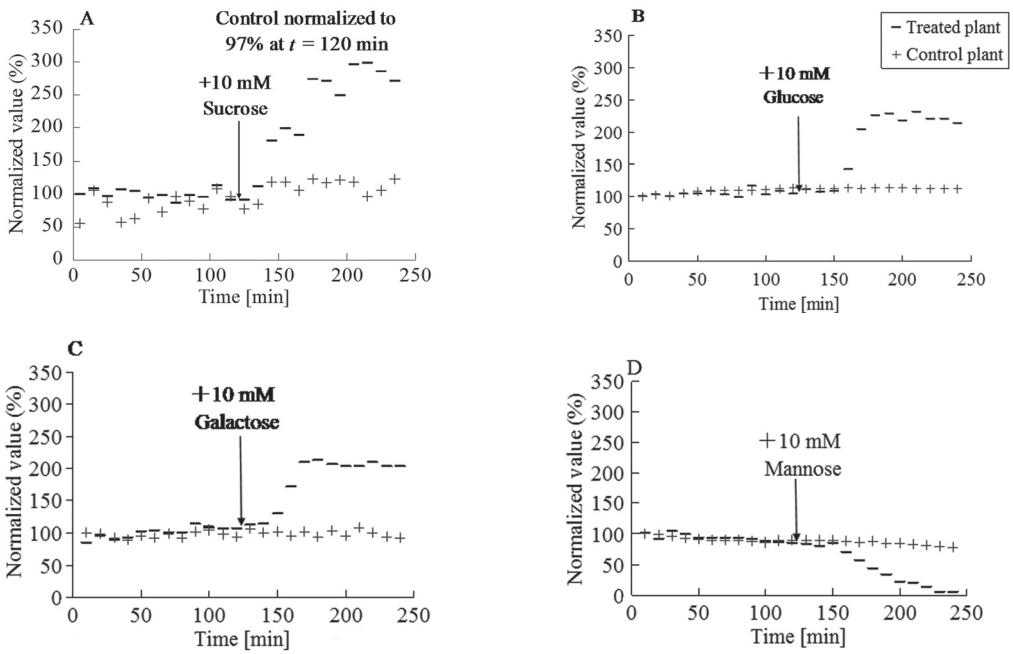


Fig. 1. The effects of sucrose, glucose, galactose, and mannose on the net nitrate uptake rate in rice roots. (A) Addition of 10 mM sucrose after 2 h. (B) Addition of 10 mM glucose after 2 h. (C) Addition of 10 mM galactose after 2 h. (D) Addition of 10 mM mannose after 2 h. Time interval is 10 min.

lasted for 1.5 h, before reaching a maximum, and the extent was 298%. By contrast, the acceleration of nitrate uptake by glucose and galactose lasted 40 min, and the nitrate uptake rates were 207% and 202%, respectively. In addition, there were obvious platform periods after the glucose and galactose treatments.

Effects of different sugars on the net nitrate uptake rate during 2 h are shown in Table I. Only sucrose had an immediate effect on the net nitrate influx; the extent of the nitrate uptake rate under sucrose treatment was the most significant, and the lag time was the shortest. Glucose, fructose, galactose, and raffinose increased the net nitrate influx only after a lag time of about 20 min. In addition, the nitrate uptake rate acceleration of these four sugars varied and was lower than that of the sucrose treatment. A platform period was also observed after the acceleration. The above result showed that sucrose could affect the nitrate uptake directly and faster than other sugars, suggesting that sucrose might be a positive feed-forward signal molecule in nitrate uptake, while lactose, mannitol, and arabinose had no significant effect on the net nitrate uptake rate in rice roots.

In addition, mannose showed inhibition of the nitrate uptake (Fig. 1D). Therefore sucrose appeared to be a specific signal molecule of nitrate uptake.

*Long-time effect of low concentration of sucrose on net nitrate uptake*

The long-time effect of 1 mM sucrose treatment on the nitrate uptake in rice roots was also measured (4 repetitions); the whole experiment lasted 11 h, and 1 mM sucrose was added after 2.5 h. The result is shown in Fig. 2.

After 1-mM sucrose treatment, the nitrate uptake of rice was accelerated immediately, and the acceleration effect was continued for 8.5 h. A platform period was not observed during acceleration.

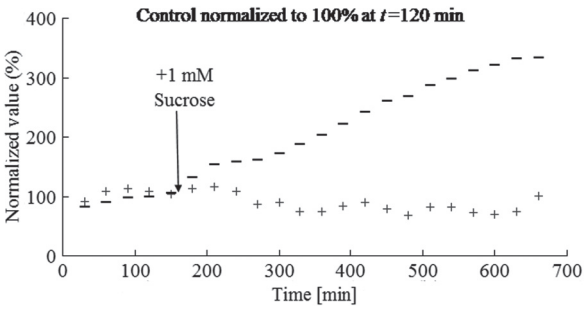


Fig. 2. The effect of 1 mM sucrose on the net nitrate uptake rate in rice roots. Time interval is 30 min.

Table I. The effects of sugars on the nitrate uptake in rice roots.

| Sugar (10 mM) | Physiological characteristics | Chemical characteristics | Lag time after addition of sugar [min] (repetition) | Increase over 2 h (extent%) (repetition) $P < 0.05$ |
|---|---|---|---|---|
| D-Sucrose | First product of photosynthesis, principal sugar transported in the phloem | Disaccharide | ≤ 5 (4) | 236 ± 57 (4) |
| D-Glucose | Metabolic intermediate, glycolysis etc. | 6-C monosaccharide | 15 ± 5 (4) | 216 ± 9.8 (4) |
| D-Fructose | Metabolic intermediate, glycolysis etc. | 6-C monosaccharide | 20 ± 5 (4) | 157 ± 49 (3) |
| D-Galactose | Cell walls | 6-C monosaccharide | 20 ± 5 (4) | 175 ± 31 (4) |
| D-Raffinose | Phloem mobile | Trisaccharide | 20 ± 10 (4) | 145 ± 18.5 (4) |
| D-Lactose | Absent | Disaccharide | No effect (3) | No effect (3) |
| D-Mannitol | Compatible solute, phloem mobile, used as non-penetrating external osmoticum | 6-C alcohol | No effect (3) | No effect (3) |
| D-Arabinose | Cell walls | 5-C monosaccharide | No effect (3) | No effect (3) |
| D-Mannose | Cell walls | 6-C monosaccharide | 30 ± 5 (3) | Decrease, 84 ± 5 (3) |

*Effects of sugars on OsNrt2.1 gene expression in rice roots*

Depending on the physiological experiment results, the effect of four sugars (three most obvious stimulating sugars, including sucrose, glucose, and galactose, and one inhibiting sugar, mannose) on the *OsNrt2.1* gene expression was measured for 2 h of treatment (Fig. 3).

Sucrose, glucose, and galactose could enhance the expression of the *OsNrt2.1* gene in rice roots. In addition, sucrose showed the strongest stimulation in the expression of the *OsNrt2.1* gene, while mannose had no effect on the *OsNrt2.1* mRNA level.

Time course changes of *OsNrt2.1* gene expression in rice roots treated with sucrose

To examine the time course changes of *OsNrt2.1* gene expression in rice roots, rice seedlings were treated with 10 mM sucrose for 8 h, and then semi-quantitative RT-PCR was carried out. It was found that the expression of the *OsNrt2.1* gene in the rice roots could be enhanced after 30 min of sucrose treatment. This enhancement continued and reached a maximum 4 h after sucrose treatment, and dropped a little bit at 8 h (Fig. 4).

## Discussion

The automated system of measuring the nitrate uptake is a novel means to obtain a continuous nondestructive measurement of the net uptake of $NO_3^-$. The system can help us to identify the detailed kinetics of nitrate uptake in a short-time scale and the variety of the lag time, which is essential to distinguish direct and indirect effects of sugars on the net nitrate influx.

The modulation of the changes in the $NO_3^-$ uptake may be a significant process involved in the co-ordination of both daily C and N acquisitions by plants. In the present experiment, we found that five sugars (Table I), including sucrose, glucose, galactose, fructose, and raffinose, could accelerate the nitrate uptake in the light. After addition of sucrose, the nitrate uptake was increased immediately (Fig. 1A), while the acceleration process remained unchanged during 8.5 h after the low-concentration sucrose (1 mM) treatment (Fig. 2). According to the result of the glucose treatment, an acceleration of nitrate uptake was also observed, but an obvious lag time between treatment and acceleration appeared; the lag time was about 20 min. We also found a similar lag and

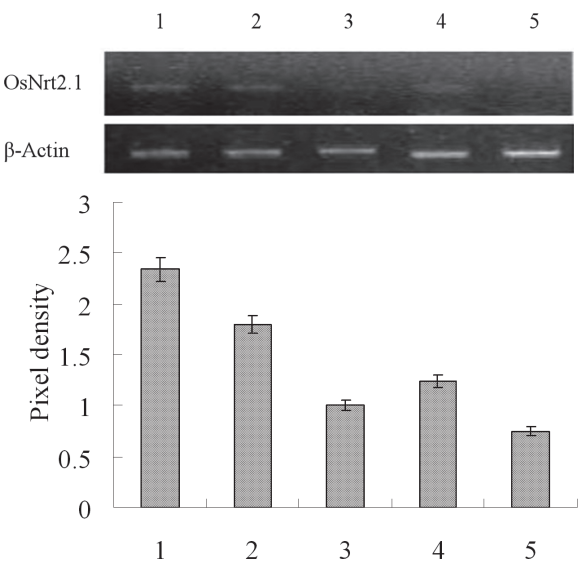


Fig. 3. The effects of 10 mM sugar treatment for 2 h on *OsNrt2.1* gene expression. 1, Sucrose; 2, galactose; 3, control; 4, glucose; 5, mannose.

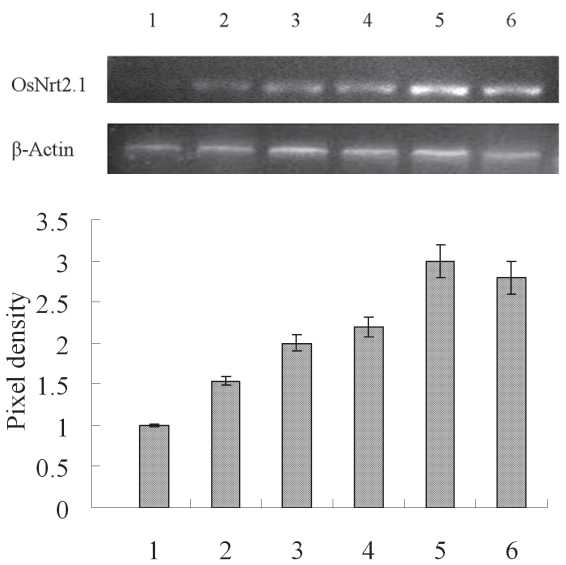


Fig. 4. The continuous effects of 10 mM sucrose treatment for 8 h on *OsNrt2.1* gene expression. 1, Control; 2, 30 min; 3, 1 h; 4, 2 h; 5, 4 h; 6, 8 h.

platform period after the galactose, fructose, and raffinose treatment, while the lag time was a little bit different. Therefore, from the results of the experiment, sucrose had the greatest effect amongst these five sugars on the nitrate uptake rate. Sugars, as important products of photosynthesis, showed close correlation with the $NO_3^-$ transport system. The relationships between sugars and $NO_3^-$ uptake have been intensively studied. Many investigators reported the sugars' positive modulation on nitrate under light/dark condition, but their studies were done on the base of long time (tens of hours to several days), the time interval was over one hour (Lejay *et al*., 1999, 2003; Sehtiya and Goyal, 2000). The question arises if the modulation could happen in a short time and the sugars can really be regarded as signals in the nitrate uptake. So it is important to care for a precise analysis of rate changes after treatment with different sugars. In the present experiment, our result was consistent with the positive sugar-signaling hypothesis. The *Nrt2.1* gene, encoding the high-affinity $NO_3^-$ transporter, is the major contributor to the inducible high-affinity $NO_3^-$ transport system (Li *et al*., 2007). The gene expression analysis showed that sucrose, glucose, and galactose enhanced the *OsNrt2.1* gene expression, among which was the most induced by sucrose. In addition, the gene expression could be obviously enhanced 30 min after sucrose treatment; the enhancement continuously increased until it reached a maximum after 4 h of sucrose treatment. Generally, the sugar can convert the formation by interconversion of sugar nucleotides in plants. So, addition of one sugar may improve the concentration of different sugars in plants and cause the same changes in gene expression. The similar effects were also found in *Arabidopsis* (Lejay *et al*., 2003). We believe that only one sugar can be the major positive signal molecule, and this sugar is sucrose.

It was shown that mannose is readily taken up by roots and converted to Man-6-P by hexokinase. However, Man-6-P is not further utilized due to a deficiency of Man-6-P isomerase that is necessary for its conversion to Fru-6-P in plants (Goldsworthy and Street, 1965). The high-levels accumulation of Man-6-P inhibits phospho-Glc isomerase, and then blocks the glycolysis metabolism (Goldsworthy and Street, 1965). The irreversible formation of Man-6-P also inhibits the respiration by depleting cells of orthophosphate required for (Goldsworthy and Street, 1965). Nitrate transport is an ATP-depending process, the treatment with mannose reduces the nitrate uptake by inhibiting the ATP production.

Thus, our data support the hypothesis that sugar signals could act as a positive feed-forward signals in the nitrate uptake. The signal molecule probably is sucrose, which is involved in the control of root nitrate acquisition and has a general significant role not only at physiological level but also at molecular level.

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