Characterization of the Arabidopsis Nitrate Transporter NRT1.6 Reveals a Role of Nitrate in Early Embryo Development

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This study of the Arabidopsis thaliana nitrate transporter NRT1.6 indicated that nitrate is important for early embryo development. Functional analysis of cDNA-injected Xenopus laevis oocytes showed that NRT1.6 is a low-affinity nitrate transporter and does not transport dipeptides. RT-PCR, in situ hybridization, and β-glucuronidase reporter gene analysis showed that expression of NRT1.6 is only detectable in reproductive tissue (the vascular tissue of the siliques and funiculus) and that expression increases immediately after pollination, suggesting that NRT1.6 is involved in delivering nitrate from maternal tissue to the developing embryo. In nrt1.6 mutants, the amount of nitrate accumulated in mature seeds was reduced and the seed abortion rate increased. In the mutants, abnormalities (i.e., excessive cell division and loss of turgidity), were found mainly in the suspensor cells at the one- or two-cell stages of embryo development. The phenotype of the nrt1.6 mutants revealed a novel role of nitrate in early embryo development. Interestingly, the seed abortion rate of the mutant was reduced when grown under N-deficient conditions, suggesting that nitrate requirements in early embryo development can be modulated in response to external nitrogen changes.

INTRODUCTION

Nitrate (NO$_3^-$) is the main nitrogen source for most plants. Plants take up nitrate from the soil using transporters in the plasma membrane of root epidermal and cortical cells. Some of the absorbed nitrate is reduced in the root by nitrate reductase, a cytosolic enzyme, some is stored in the vacuole of root cells, and some is transported to the shoot for assimilation or storage. Nitrate must cross several membrane systems to be distributed in the different tissues and organelles.

Two families of nitrate transporters, NRT1 and NRT2, have been identified in higher plants. NRT1s belong to the NRT1(PTR) family, containing some members that have been shown to be nitrate transporters (NRT1) and some to be dipeptide transporters (PTR) (reviewed in Tsay et al., 2007). In Arabidopsis thaliana, there are 53 NRT1(PTR) genes and seven NRT2 genes. The nitrate transporters in the NRT1(PTR) family were initially thought to transport nitrate with low affinity, until one, Arabidopsis NRT1.1 (also known as CHLorate resistant 1 CHL1), was found to be a dual-affinity transporter involved in both low- and high-affinity nitrate uptake (Wang et al., 1998; Liu et al., 1999; Liu and Tsay, 2003). However, all other members of the Arabidopsis NRT1 family so far examined, such as NRT1.2 (involved in nitrate uptake), NRT1.4 (involved in petiole nitrate storage), and NRT1.5 (involved in xylem loading), are pure low-affinity transporters (Huang et al., 1999; Chiu et al., 2004; Tsay et al., 2007). No sequence homology exists between the NRT1 and NRT2 families. Two members of the plant NRT2 family characterized in Xenopus laevis oocytes, Hv NRT2.1 and At NRT2.1, were shown to be high-affinity nitrate transporters (Tong et al., 2005; Orsel et al., 2006). Two of the NRT2 genes in Arabidopsis, NRT2.1 and NRT2.2, are involved in the inducible phase of high-affinity nitrate uptake (Cerezo et al., 2001; Filleur et al., 2001; Li et al., 2007), while NRT2.7 is involved in seed nitrate storage (Chopin et al., 2007).

During vegetative growth, inorganic nitrogen, such as nitrate, is the major source of nitrogen, but organic nitrogen, such as amino acids and peptides, is thought to be the major nitrogen source for seed development and germination. The roles of amino acids and peptides in seed development have been documented by studies of amino acid transporters and peptide transporters. Three amino acid transporters from the amino acid permease (AAP) family, AAP1, 2, and 8, are expressed in reproductive organs. In Arabidopsis siliques, AAP2 is expressed only in the vascular system, including the funiculi, AAP1 is only expressed in the seed, while AAP8 is expressed in both the vascular system and seed (Hirner et al., 1998; Okumoto et al., 2002). It has been suggested that AAP8 and AAP1 are involved in amino acid import for embryo growth or storage protein synthesis, respectively (Hirner et al., 1998; Schmidt et al., 2007). In addition, ectopic expression of Vicia faba AAP1 in pea (Pisum sativum) and in Vicia narbonensis leads to an increase in protein content in the seeds (Rolletschek et al., 2005). The study of these AAP transporters indicates that seed amino acid transport activity is important for embryo development and storage protein accumulation.
In barley (Hordeum vulgare), during seed germination, proteins stored at the end of seed development are broken down and remobilized by the dipeptide transporter PTR1 (West et al., 1998; Waterworth et al., 2005). In addition, during embryo development, several dipeptide transporters (PTRs) (Rentsch et al., 1995; Song et al., 1996, 1997; Miranda et al., 2003; Dietrich et al., 2004) and oligopeptide transporters (OPTs) (Stacey et al., 2002, 2006) are expressed. Although an antisense PTR2 line and a mutant opt3 line show an embryo lethal phenotype in Arabidopsis (Song et al., 1997; Stacey et al., 2002), new evidence (Wintz et al., 2003, Tsay et al., 2007), described in the Discussion indicates that further studies are needed to clarify whether peptide transport is important for embryo development.

In addition to organic nitrogen, nitrate can be accumulated in seeds and affect seed dormancy (Alboresi et al., 2005). A recent study (Chopin et al., 2007) showed that Arabidopsis NRT2.7, a member of the NRT2 family, is involved in nitrate accumulation in seeds and that NRT2.7 is located in the tonoplast and is expressed in developing seeds, particularly at the end of seed maturation. In the nrt2.7 mutant, the nitrate content of mature seeds was reduced, and fresh harvested seeds were more dormant than wild-type seeds. The above study shows that NRT2.7 affects the amount of nitrate accumulated in seeds. Nevertheless, it is still not known whether nitrate influences seed development. In this study, by characterization of the Arabidopsis nitrate transporter NRT1.6, we show that nitrate affects seed development at a very early stage and that the requirement for nitrate in seed development is modulated by the external nitrogen supply.

RESULTS

Cloning of Arabidopsis NRT1.6 cDNA

NRT1.6 (At1g27080) is one of the 53 NRT1(PTR) genes in Arabidopsis. In a search of the databases, no EST clone was found and three different gene structures were predicted. To validate the gene structure, RT-PCR was performed using genespecific primers (see Methods). Sequence analysis of the PCR fragment obtained revealed that there were, in fact, four exons and three introns (see Supplemental Figure 1A online) and that none of the predicted structures was correct (see Supplemental Figure 1B online). Hydropathy analysis showed that the encoded 576-amino acid protein contained 12 putative transmembrane domains with an intracellular central loop, the typical topology of the NRT1 transporters. The predicted protein sequence of NRT1.6 shows 32, 34, 36, and 34% identity with that of At NRT1.1 (CHL1), At NRT1.2, At NRT1.4, and At NRT1.5, respectively (see Supplemental Figure 2 online).

NRT1.6 Is a Nitrate Transporter

Some members of the NRT1 family (e.g., CHL1, Bn NRT1.2, At NRT1.2, At NRT1.4, and Os NRT1) (Tsai et al., 1993; Zhou et al., 1998; Huang et al., 1999; Lin et al., 2000; Chiu et al., 2004) are nitrate transporters, and some (e.g., At PTR1, At PTR2, and Hv PTR1) are di/tripeptide transporters (Song et al., 1997; West et al., 1998; Dietrich et al., 2004). A functional assay was therefore performed on NRT1.6 complementary RNA (cRNA)-injected Xenopus oocytes to determine whether NRT1.6 is a nitrate transporter or peptide transporter. In NRT1.6-injected oocytes, an inward current was elicited by nitrate at pH 5.5 (Figure 1A), indicating that NRT1.6 is a nitrate transporter. The peptide transporters in the NRT1(PTR) family are known to take up dipeptides without excluding any of the different amino acid compositions examined (Chiang et al., 2004; Tsay et al., 2007). Because no current was elicited by dipeptides GlyGly and LeuLeu in NRT1.6-injected oocytes, in combination this suggests that NRT1.6 is not a peptide transporter. As expected for a proton-coupled nitrate transporter with a proton/nitrate ratio larger than one, in NRT1.6-injected oocytes, an inward current was elicited by the negatively charged nitrate (Figure 1A) and the current elicited by nitrate was pH dependent (Table 1).

One member of the NRT1 family, At NRT1.1 (CHL1), is a dual-affinity nitrate transporter with a $K_m$ of $\sim 50\mu$M for its high-affinity mode and $\sim 5$ mM for its low-affinity mode (Liu et al., 1999), but all other characterized NRT1 transporters (At NRT1.2, At NRT1.4, and Os NRT1) are pure low-affinity nitrate transporters (Huang et al., 1999; Lin et al., 2000; Chiu et al., 2004). To determine the uptake properties of NRT1.6, the nitrate transport activities of cRNA-injected oocytes were measured by incubating the cells with 0.25 mM (to assess level of high-affinity uptake, Figure 1B) or 10 mM (to assess level of low-affinity uptake, Figure 1C) $^{15}$N-labeled nitrate. Consistent with the previous data, CHL1 cRNA-injected oocytes showed nitrate transport activities at either 0.25 or 10 mM nitrate, while NRT1.6 cRNA-injected oocytes showed transport activity only at 10 mM (Figure 1C).

Low-affinity transport activity of NRT1.6 was further confirmed by kinetic analysis of currents elicited by different concentrations of nitrate. The $K_m$ of NRT1.6 for nitrate, calculated by fitting the currents elicited at $-80$ mV by different concentrations of nitrate to the double reciprocal equation, was $\sim 6$ mM (Figure 1D). Taken together, these functional analyses in Xenopus oocytes indicate that NRT1.6 is a pure low-affinity nitrate transporter with a $K_m$ of $\sim 6$ mM.

NRT1.6 Expression in Siliques Increases after Pollination

RT-PCR analysis revealed that NRT1.6 is only expressed in flowers and siliques (Figure 2A). To determine the temporal pattern of NRT1.6 expression, RNA was prepared from flowers and siliques at different stages of development. Flower stages were defined as follows: stage A, closed flowers; stage B, opening flowers; stage C, 3-mm-long siliques in which pollination has just occurred; and stage D, 4- to 5-mm-long siliques (2 to 4 d after pollination, containing seeds with embryos at the 2-cell or 8-cell stage). Stages A, B, C, and D correspond to flower stages 11-12, 13, 14, and 15-16, respectively, defined by Smyth et al. (1990). As shown in Figure 2B (data points shown as circles), quantitative RT-PCR showed a low level of NRT1.6 transcript in closed and opening flowers (stages A and B), and that expression increased dramatically after pollination (stage D). Since NRT1.6 is a nitrate transporter, it was of interest to determine whether nitrogen deficiency affected NRT1.6 expression. As shown in Figure 2B (data points shown as diamonds),...
when plants were grown under nitrogen-deficient conditions, the temporal pattern of \textit{NRT1.6} expression was not altered, but the level of expression at stage D increased compared with normal conditions, indicating that nitrogen starvation induces expression of \textit{NRT1.6}.

The spatial distribution of \textit{NRT1.6} expression in siliques was examined by RNA in situ hybridization. Compared with the sense control, \textit{NRT1.6}-specific signals were found in the funiculus of the sections hybridized with the antisense probe (Figures 3A to 3D). High-density signals in embryo sac wall and the epidermis of siliques were found in the sections hybridized with either the antisense probe (Figures 3A and 3B) or the sense probe control (Figures 3C and 3D), indicating that the signals in embryo sac wall and the epidermis of siliques were nonspecific background signal.

To confirm that \textit{NRT1.6} is expressed in funiculus, \textit{\textbeta}-glucuronidase (GUS) activity in a transgenic line harboring a GUS-containing T-DNA inserted in the first intron of \textit{NRT1.6} was analyzed. As shown in Figures 3E and 3F, GUS staining was seen in the vascular bundle of the siliques and in the funiculus, suggesting that \textit{NRT1.6} is involved in delivering nitrate for seed development.

Subcellular Localization of \textit{NRT1.6} Protein

To investigate the subcellular localization of \textit{NRT1.6}, a fusion protein of green fluorescent protein (GFP) fused C-terminally to \textit{NRT1.6} (\textit{NRT1.6-GFP}) was expressed in \textit{Arabidopsis} protoplasts under the control of the cauliflower mosaic virus 35S RNA promoter. GFP staining was seen in the cytoplasm in the GFP control (Figure 3G, bottom panels), while the fusion protein was detected as a fine ring at the cell periphery, external to the chloroplasts (Figure 3G, top panels), indicating that \textit{NRT1.6} is localized in the plasma membrane.

\textit{nrt1.6} Knockout Mutants Show Increased Seed Abortion

Three independent \textit{nrt1.6} mutant lines were used in this study (Figure 4A). Mutant \textit{nrt1.6-1} contained a T-DNA with the GUS gene inserted in the first exon, \textit{nrt1.6-2} contained a single T-DNA inserted in the third intron, and \textit{nrt1.6-3} contained two copies of T-DNA in opposite directions inserted in the second and fourth exons. The \textit{NRT1.6} transcript was weakly expressed in the \textit{nrt1.6-1} mutant, but not in the other two (Figure 4B; see Figure 1. Nitrate Uptake Activity of \textit{NRT1.6} cRNA-Injected Oocytes.

(A) Substrate specificity of \textit{NRT1.6}. Oocytes were voltage-clamped at \(-60\) mV and stepped to a test voltage between 0 mV and \(-140\) mV for 300 ms, in \(-20\)-mV increments. The currents (\(i\)) shown here are the difference between the currents flowing at \(+300\) ms in the presence and in the absence of 10 mM nitrate or dipeptides at pH 5.5. Similar results were obtained from three other oocytes from different frogs.

(B) and (C) Nitrate uptake activity of \textit{NRT1.6}- or \textit{CHL1}-injected oocytes.

(D) Kinetics of nitrate-elicited currents in a single \textit{NRT1.6} cRNA-injected oocyte. The oocyte was voltage clamped at \(-60\) mV. The inward current elicited by different concentration of nitrate at pH 5.5 is plotted as a function of the external nitrate concentration. The inset shows a Lineweaver-Burk plot. The example shown is representative of the results from nine oocytes from three different frogs.
Embryo Abnormalities in the nrt1.6 Knockout Mutants

To characterize the process of seed abortion, wild-type and mutant embryos at various stages of seed development were analyzed. When wild-type seed was at the globular stage of development, 20 to 30% of the seeds from the mutant siliques were found to be shriveled (compared with 2% in the wild type) and the tissues inside collapsed. Aberrant embryo development in the mutant could be seen as early as the one-cell and four-cell stages of normal development.

The frequency of abnormal embryos in the nrt1.6 mutants and wild type grown in the same pot was counted. The seeds from these siliques were at the one- to eight-cell stages of development. As shown in Figure 5A, the percentage of abnormal embryos in the mutant (~40%) was 4 to ~6 times higher than that in the wild type (~7%). In the mutants, the percentage of abnormal embryos (~40%) observed at one- to four-cell stages was higher than the percentage of seed abortion (~20%) observed at the walking-stick stage, suggesting that some of the abnormal embryos were able to develop into normal seeds.

As expected for normal embryo development, the wild-type zygotes underwent asymmetric division to create two cells (Figure 5B), a small, round apical cell, which formed the embryo after several divisions, and a long basal cell, which underwent transversal divisions to generate a filament of suspensor cells (Figure 5C). At the one-cell stage in the wild type, there was usually only one suspensor cell (Figure 5B). In the mutant, one of the abnormalities found at the one-cell stage was a suspensor with multiple cells (Figure 5D), which was seen in ~50% of the aberrant embryos. Another abnormality found in the mutant embryos at the one-cell to four-cell stages was an irregular shape of the suspensor cells, which were collapsed or twisted (Figures 5E and 5F); this was seen in ~40% of the aberrant embryos. An additional abnormality found at this stage was two enlarged cells at the tip, rather than one (Figure 5G); this was seen in ~5% of abnormal embryos. It is not clear whether this is due to a wrong

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**Table 1. Inward Current (nA) Elicited in Oocytes Injected with NRT1.6 cRNA or Water and Exposed to 10 mM Nitrate at the Indicated pH**

<table>
<thead>
<tr>
<th>Oocyte Injection</th>
<th>pH 5.5</th>
<th>pH 7.4</th>
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<tbody>
<tr>
<td>NRT1.6</td>
<td>26.6 ± 5.0</td>
<td>8.3 ± 2.8</td>
</tr>
<tr>
<td>Water</td>
<td>1.6 ± 2.8</td>
<td>6.6 ± 2.8</td>
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Oocytes were voltage clamped at ~60 mV using two microelectrodes. The results are the average for six oocytes from three frogs.

Supplemental Figure 4 online), so the nrt1.6-1 line was used for the GUS-staining assays shown in Figures 3C and 3D. The two mutant lines nrt1.6-2 and nrt1.6-3, with no detectable NRT1.6 expression (Figure 4B), were used for the phenotype study below.

To determine whether the nrt1.6 mutation had any effect on seed development, the siliques were dissected. In the mutant siliques, some seeds remained small, transparent, and shriveled, while other seeds developed normally and were green and swollen (Figure 4C). To compare the seed abortion rates, nrt1.6 mutants and wild-type plants were grown in the same pot. As shown in Figure 4D, when grown under normal conditions, the percentage of aborted seeds in the nrt1.6-2 and nrt1.6-3 mutants (examined at the walking-stick stage, 4 to 5 d after flowering) was ~13 times higher than in wild-type plants. Supplying an extra 10 mM nitrate to our regular fertilizer didn’t change the abortion rate in either the wild type or mutant, suggesting that the nitrogen in the regular fertilizer is sufficient for seed development; increasing external nitrate cannot rescue the abortion phenotype of the mutants. To study the effect of nitrogen starvation on seed abortion, wild-type and mutant plants were deprived of nitrogen for 1 week before bolting. As shown in Figure 4D, as expected, the percentage of aborted seeds in the wild type increased slightly. However, in the mutants, the percentage was dramatically less than under normal conditions.

The aborted seeds in the mutant were randomly distributed along the siliques, and the total number of possible seeds per siliques (normal seeds plus aborted seeds) was similar in the wild type and mutant. In addition, no differences were found between the wild type and mutants in the length of the siliques at various stages of development, the weight of the mature seeds, or the percentage germination of the nonaborted seeds.

Figure 2. NRT1.6 Expression in the Siliques and Seeds of Arabidopsis.

(A) RT-PCR products of NRT1.6 (30 cycle-amplified) and UBQ10 (20 cycle-amplified). RNA was isolated from different plant tissues. Shoot (S) and root (R) tissues of plants grown for 14 d in NH₄NO₃. Inflorescence stem (St), cauline leaves (Cl), flower and flower buds (Fl), and siliques (Sl) from 4-week-old plants grown in soil. Et, 5-d etiolated seedling.

(B) Quantitative PCR. RNA isolated from siliques in different stages of development was used for quantitative PCR. Stage A, closed flower; stage B, 3 mm long; and stage D, 5 mm long. Plants were grown under normal (circles) or nitrogen-limited conditions (diamonds). The relative expression level is the expression normalized to the expression of UBQ10. Values are means ± SD of three biological repeats. * Significant difference at P < 0.05 compared with the level in stage B. # Significant difference (P < 0.05) between normal and nitrogen-limited conditions.
division in the embryo cell or an enlargement of the top suspensor cell. In the mutants, abnormalities were mainly found at the one- and four-cell stages, but all embryos which reached the octant stage were normal, suggesting that aborted embryos are arrested before or at the four-cell stage.

Seed Nitrate Content Is Reduced in the nrt1.6-2 Mutant

The nitrate content of seeds is highly dependent on the external nitrate supply (Alboresi et al., 2005). To avoid differences due to soil nitrate availability, the nitrate content of the seeds from wild-type and mutant plants grown in the same pot was compared. Seeds from six pairs of plants were collected for nitrate content analysis. As shown in Figure 6, when plants were grown under normal conditions, the nitrate content of wild-type seeds fluctuated from plant to plant, but, in all of them, the content was higher (up to eightfold) than in the nrt1.6-2 mutant. As expected, when plants were starved of nitrogen, the nitrate content of both sets of seeds was reduced to a very low level, with no obvious difference between the wild type and the mutant.

DISCUSSION

Arabidopsis NRT1.6 Is Expressed in the Funiculus and the Vascular Tissue of the Silique and Is Involved in Delivering Nitrate for Seed Development

Functional analysis in Xenopus oocytes showed that NRT1.6 is a pure low-affinity nitrate transporter, with a $K_m$ measured in oocyte of $\sim 6$ mM. RT-PCR showed that NRT1.6 transcripts were detected in the flower and silique, but not in any of the vegetative tissues tested. In situ hybridization and GUS staining showed that NRT1.6 was expressed in the funiculus of the seeds and the vascular bundle of siliques. Mutant lines lacking NRT1.6 expression were deficient in seed nitrate accumulation. Taken together, these data suggest that NRT1.6 is involved in the delivery of nitrate to the developing seed.

By studying the movement of GFP in Arabidopsis seeds, Stadler et al. (2005) showed that the outer integument is a symplastic extension of the funicular phloem, suggesting that nutrients at the end of the funiculus can be unloaded through the outer integument. Like NRT1.6, the Arabidopsis amino acid transporter AAP2 (Himer et al., 1998) and the Arabidopsis sucrose transporter SUC2 (Truernit and Sauer, 1995) are also expressed in the vascular tissue of the silique and the funiculus, indicating this is a common path for the delivery of nutrients to developing seeds. Due to the symplastic continuum formed between funiculus and outer integument, expression of NRT1.6, AAP2, and SUC2 in the funiculus would suggest that these

Figure 3. NRT1.6 Is Expressed in Funiculus, and the Protein Is Located in the Plasma Membrane.

(A) and (B) In situ hybridization of the antisense NRT1.6 probe to a section of Arabidopsis silique.

(C) and (D) In situ hybridization of the sense NRT1.6 probe to a section of Arabidopsis silique. Green arrows indicate the signals in funiculus (F). White triangles indicate the nonspecific signals in embryo sac wall and the epidermis of silique, which were found in sections hybridized with either antisense or sense probe.

(E) and (F) Localization of GUS activity (blue) in the mutant line nrt1.6-1.

(E) shows a longitudinal section of the silique. The area labeled with white box was enlarged and is shown in (F). The seed coat appears orange in this section. F, funiculus; VB, vascular bundle.

(G) Subcellular localization of NRT1.6-GFP fusion protein in Arabidopsis protoplasts. Confocal laser scanning microscope pictures (left) and corresponding bright-field images (right) of Arabidopsis protoplasts transiently expressing NRT1.6-GFP (top panels) or GFP alone (bottom panels).
transporters may function as importers to accumulate nutrient in the funiculus to be delivered to the outer integument. However, in contrast with NRT1.6, which was found to be only expressed in reproductive tissue, AAP2 and SUC2 were also expressed in the vascular bundle of vegetative tissue. With 53 NRT1(PTR) genes in the Arabidopsis genome, some NRT1 genes might have a specific function in nitrate or nitrogen distribution.

Role of Nitrate in Early Embryo Development

Organic nitrogen (amino acids and peptides) is known to be the major form of nitrogen delivered from maternal tissue to the developing seeds. However, the nrt1.6 null mutation showed suspensor abnormalities at the one-cell and four-cell stages of embryo development, which led to a high percentage of seed abortion, suggesting that inorganic nitrogen (nitrate) is also important in embryo development.

The seed abortion rate of nrt1.6 was ~20%, suggesting that some seeds could bypass the requirement for nitrate or overcome the defects caused by nitrate shortage at early stages of development. Alternatively, some other NRT1 transporter may be functionally redundant to NRT1.6 in providing nitrate for early embryo development. Available public microarray data and our RT-PCR analysis (Tsay et al., 2007) indicated that nine NRT1(PTR) genes, At1g27040, At1g69860, At1g69870, At2g02040, At3g16180, At3g25260, At4g21680, At5g01180, and At5g46040, were expressed in the silique. To find out if some of these genes were upregulated to compensate for the lost function of nrt1.6 in embryo development, expression levels of these nine NRT1 genes in the wild type and nrt1.6-2 mutant were analyzed and compared. However, none of these genes were upregulated in the nrt1.6-2 mutant (data not shown). Therefore, further study is required to find out if any of these NRT1 genes is functionally redundant to NRT1.6.

In the embryo of nrt1.6 mutants, abnormalities were mainly found in the suspensor. Up to ~20% of the suspensors in the mutants at the one-cell to four-cell stage were either twisted or collapsed, presumably due to loss of turgidity. As a consequence, some of the embryos (~2%) were not held in the proper position and were found to be hanging out of the micropyle (see Supplemental Figure 3 online). Another abnormality found in the

Figure 4. Seed Abortion Rate Is Increased in nrt1.6 Mutants.

(A) T-DNA insertion sites in the NRT1.6 coding region in the three different mutant lines used in this study. Mutants nrt1.6-1 and nrt1.6-2 had a single T-DNA inserted in the first or third introns, respectively, while two copies of T-DNA were inserted in the second and fourth exons of nrt1.6-3. The black boxes represent exons and the arrowheads indicate T-DNA left border primers. F, forward primer used for RT-PCR; R, reverse primer used for RT-PCR.

(B) NRT1.6 expression in mutant lines. Total RNA from siliques of the wild-type (1) or the nrt1.6-2 (2) or nrt1.6-3 (3) mutant was used for RT-PCR analysis. Control reactions (UBQ10) were amplified for 18 cycles, while NRT1.6 samples were amplified for 30 cycles.

(C) Silique from an nrt1.6-2 plant containing normal green seeds (black arrowheads) and aborted seeds (white arrowheads).

(D) Quantification of seed abortion in the nrt1.6 mutants and the wild type. Plants were grown under normal (Regular) or nitrogen-deficient (Starved) conditions and the percentage of aborted seeds calculated. Values are means ± SE of 18 to 30 siliques from three independent plants. The possible seeds (normal seeds plus aborted seed) per siliques were ~45 to 55, which was not significantly different between the wild type and mutant. *, P < 0.05 compared with the wild type.
mutant was multiple suspensor cells at the one-cell stage, in contrast with the single suspensor cell seen in the wild type at this stage. This may be due to excessive cell division of the suspensor at the one-cell stage or to a delayed development of the embryo proper in these seeds. The latter possibility is less likely, as we always compared wild-type and mutant seeds from siliques at the same stage of development and the percentages of embryos at the one-cell and four-cell stages in the wild type and mutants were similar. Thus, the two major defects found in the mutant suspensor were loss of turgidity and excessive cell division; whether these two defects are physiologically linked or independent events remains to be determined.

The embryo of the nrt1.6 mutants was arrested at or before the four-cell stage, suggesting that nitrate is required for the earlier stages of embryo development. Study of the cell-to-cell movement of GFP showed that, at the globular stage, the embryo and suspensor form a symplastic continuum (Kim and Zambryski, 2005; Stadler et al., 2005). However, in the nrt1.6 mutants, loss of turgidity was only seen in the suspensor. If the loss of turgidity observed in the suspensor is due to an osmotic role of nitrate, the absence of abnormalities in the embryo proper conflicts with the symplastic continuum model. One possibility is that, at earlier stages (one-cell and two-cell), the symplastic connection between the embryo proper and suspensor has not yet been established.

Comparison of Embryo Abnormalities in Nitrogen Transport Mutants

Several Arabidopsis genes involved in the transport of organic nitrogen into the seeds have been identified, and mutants of
some of these show arrested embryogenesis. The amino acid transporter AAP8 is one such case. In the aap8 mutant, ~45% of the seeds are aborted at, or before, the globular stage (Schmidt et al., 2007). Abortion in the nrt1.6 mutant also occurred at a very early stage. One difference between the mutants of AAP8 and NRT1.6 is the total number of possible seeds (normal seeds plus aborted seeds) per silique, which is reduced by 50% in aap8, but was unchanged in the nrt1.6 mutant.

In addition to AAP8, mutants of two Arabidopsis peptide transporters also show embryo defect phenotypes. Mutation in the peptide transporter OPT3 is embryo-lethal, and embryos of the opt3 mutant are arrested at, or before, the octant stage (Stacey et al., 2002). Interestingly, in contrast with the nrt1.6 mutants, the opt3 mutant shows normal suspensor development. However, yeast complementation showed that OPT3 can transport metals (Wintz et al., 2003) and whether the embryo defect is linked to the metal transport activity or the potential peptide transport activity of OPT3 remains to be clarified. In addition, antisense lines of the Arabidopsis dipeptide transporter PTR2 show a seed abortion rate of 50 to 60% (Stacey et al., 2002), but a T-DNA–inserted mutant of PTR2 shows normal seed development (G. Stacey, personal communication), suggesting that some unknown member(s) of the NRT1(PTR) family is cross-sensitized in the antisense lines. However, we checked the expression of the NRT1.6 gene in the ptr2 antisense lines and found no difference compared with the wild type, showing that NRT1.6 is not responsible for the seed abortion observed in ptr2 antisense lines. Studies of the barley peptide transporter Hv PTR1 showed that peptide transport is important for seed germination (West et al., 1998; Waterworth et al., 2005). Whether peptide transport is important for embryo development is an open question.

The Role of Nitrate in Embryo Development Is Modulated by External Nitrogen

In the nrt1.6 mutants, the seed abortion rate was high (~20%) when grown under normal conditions, and surprisingly, was reduced (~7%) when grown under conditions of nitrogen deficiency, indicating that, under deficiency conditions, the requirement for nitrate in early embryo development can be alleviated and probably compensated by some other compound or pathway. Indeed, using the same RNA samples as shown in Figure 2B, AAP1 was found to be upregulated by nitrogen deficiency (see Supplemental Figure 5 online), suggesting that the plant can sense the nitrogen status and modulate its nutrition strategy for grain development.

Under normal conditions, the seed nitrate content was inversely correlated with the seed abortion rate. In the wild type, the seed nitrate content was high and the abortion rate low, while, in the mutant, the seed nitrate content was low and the abortion rate high. Although the nitrate content measured may mainly reflect nitrate stored at the end of seed development, the reduced nitrate content in the mutant may imply that less nitrate is transported into the seed at the early stage of embryo development. Under nitrogen-deficient conditions, the seed nitrate content of the wild type was reduced to the mutant level, but the seed abortion rate was only slightly increased compared with that under normal conditions. This further supports the hypothesis that nitrate requirements in early embryo development are modulated by the external nitrogen status. Modulation of nutrient strategy for embryo development would minimize the grain yield lost under mild nutrient deficiency conditions.

METHODS

Plant Growth Conditions and Seed Collection

Plants were grown at 22°C under continuous light and irrigated with HYPONeX #2 fertilizer (20% N including 4% nitrate and 4% ammonium, 20% P2O5, and 20% K2O [HYPONeX]) at final concentrations of 6 mM nitrate, 5.3 mM K, and 3.5 mM P. For nitrogen starvation treatment, 3-week-old plants were watered with a solution containing 5 mM K2HPO4/KH2PO4, pH 5.5, 1 mM MgSO4, 0.1 mM FeSO4·EDTA, 0.5 mM CaCl2, 50 μM H3BO3, 12 μM MnSO4, 1 μM ZnCl2, 1 μM CuSO4·H2O, and 0.2 μM Na2MoO4·2H2O. Seed abortion, seed nitrate content, and embryo development were compared between wild-type and mutant plants grown in the same pot.

Functional Expression of NRT1.6 in Xenopus laevis Oocytes

NRT1.6 cDNA was subcloned into the pGEMHE vector (Liman et al., 1992) and linearized using NheI. Capped mRNA was then transcribed in vitro using mMessage mMachine kits (Ambion). Oocytes from Xenopus frogs were injected with 100 ng of cRNA as described previously (Tsay et al., 1993). Electrophysiological analyses of injected oocytes were performed as described previously (Huang et al., 1999). Nitrate uptake assays were performed using 15N-labeled nitrate, and oocytes injected with CHL1 cRNA (Li et al., 1999) were used as a positive control. Oocytes were incubated for 2 h in a solution containing 230 mM mannitol, 0.3 mM CaCl2, 10 mM Mes-Tris, pH 5.5, and the required concentration of K15NO3 (0.25 mM for high-affinity uptake assays and 10 mM for low-affinity uptake experiments), rinsed five times with ND96 buffer (Broer et al., 2002), but a T-DNA–inserted mutant of PTR2 shows normal seed development (G. Stacey, personal communication), suggesting that some unknown member(s) of the NRT1(PTR) family is cross-sensitized in the antisense lines. However, we checked the expression of the NRT1.6 gene in the ptr2 antisense lines and found no difference compared with the wild type, showing that NRT1.6 is not responsible for the seed abortion observed in ptr2 antisense lines. Studies of the barley peptide transporter Hv PTR1 showed that peptide transport is important for seed germination (West et al., 1998; Waterworth et al., 2005). Whether peptide transport is important for embryo development is an open question.

Two Arabidopsis Nitrate Transporter Genes Are Involved in Nitrate Storage in Seeds

Our study of nrt1.6, here, and the known properties of nrt2.7 indicate that nitrate is required for two different stages of embryo development in Arabidopsis. First, at the one- to four-cell stage, nitrate is required for proper embryo development and NRT1.6 is involved in delivering nitrate to fulfill the nitrate requirements at this stage. Abnormal embryos are found in nrt1.6 mutants (this study) but not nrt2.7 mutants (Chopin et al., 2007), indicating that NRT2.7 is not required at this early stage of embryo development. Indeed, high expression of NRT2.7 is found only at the late stage of seed maturation.

Second, at the end of seed development, nitrate is probably stored in the vacuoles of the cotyledons and radicles, and nitrate stored at this stage will affect seed dormancy (Chopin et al., 2007). The reduced seed nitrate content of the nrt1.6 and nrt2.7 mutants indicates that NRT1.6 is also involved in delivering nitrate from vascular tissue for seed storage, while NRT2.7, located in the tonoplast of the cotyledon and radicles and expressed in the very late stage of embryo development, is involved in pumping nitrate into the vacuole. NRT1.6 and NRT2.7 may therefore work together to ensure that nitrate is stored in seeds.
et al., 2000), and individually dried at 80°C for 24 h, and then the retained 15N was analyzed on a continuous-flow isotope ratio mass spectrometer coupled with a carbon nitrogen elemental analyzer (ANCA-GSL MS; PDZ Europa).

RT-PCR and Quantitative PCR

First-strand cDNAs were synthesized using RNA isolated from different developmental stages of flowers and siliques and ImProm-II reverse transcriptase (Promega) and oligo(dT) primers. Primers specific for the NRT1.6 or control UBQ10 gene were then used to amplify each transcript using 18 PCR cycles (UBQ10) or 35 cycles (NRT1.6). The primers used were as follows: NRT1.6 (forward, 5'-ATGGAGATTGTTGAGAATCG-3'; reverse, 5'-CTAAGTTTGGAGATTTGACG-3') and UBQ10 (forward, 5'-GGATCTCACTGCGGACG-3'; reverse, 5'-CTCCTTAAGCATACAAGACGG-3').

Quantitative PCR was performed in a LightCycler PCR using Power SYBR Green (ABI System). The primers used for quantitative PCR were as follows: NRT1.6 (forward, 5'-CAATTCAGATTCTGGAACAACT-3'; reverse, 5'-GACCTCTTCAACTTCTTGATGCTA-3') and UBQ10 (forward, 5'-AGAAAGTCTAGTTGATCGTGTA-3'; reverse, 5'-GAACCGAACATAGTAGAACACTT-3').

In Situ Hybridization

Plants were grown in the soil for 1 month, and ~5 mm siliques were harvested for fixation and processed as described by Huang et al. (1999). 35S-labeled NRT1.6 antisense (or sense) RNA was synthesized using SP6 (or T7) RNA polymerase from Ncol (or NdeI)-linearized pTe-NRT1.6 (pGEM-T Easy [Promega] containing the 1.6-kb coding region of NRT1.6 cDNA). Silique sections were hybridized with the hydrolyzed RNA probes at 45°C and washed as described previously (Huang et al., 1999), and the slides were coated with Kodak NTB-2 emulsion (International Biotechnologies) and exposed for 1 month at 4°C.

GUS Analysis of Transgenic Plants

The nrt1.6-1 T-DNA-inserted mutant, obtained from the Wisconsin Knockout Facility, contains the GUS gene in its T-DNA. Siliques were stained for GUS using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as substrate according to a published protocol (Jefferson et al., 1987). Stained siliques in 50% ethanol were placed under vacuum for 2 × 15 min and then incubated at atmospheric pressure and room temperature (RT) for 2 h. The specimens were then dehydrated at RT for 3 × 30 min in each of various concentrations of ethanol (70, 95, and 100%). After overnight incubation at RT in 100% ethanol, the specimens were embedded using 100% ethanol-xylene (1:1) for 15 min at RT, pure xylene for 2 × 15 min at RT, xylene-melted Paraplast (Oxford) (3:1, 1:1, and 1:3; 30 min each at 62°C), and pure melted Paraplast for 3 × 1 h at 62°C, and then tissues embedded in the desired orientation were cut into 8-μm sections.

GFP Fusion and Subcellular Localization

To construct the NRT1.6-GFP fusion protein, NRT1.6 cDNA was amplified by PCR using the primers NRT1.6F (5'-GAGTTGTTGAGAAT-3') and NRT1.6R (5'-CCCGGATCCTGCGGACG-3'), the latter of which removes the stop codon and introduces a BamHI restriction site. The amplified DNA fragment was then cloned in frame in front of the GFP coding region in the vector 326-GFP (Lee et al., 2001), leading to the final NRT1.6-GFP construct under the control of the 35S promoter. The NRT1.6-GFP fusion construct or the vector 326-GFP was transiently expressed in Arabidopsis thaliana protoplasts.

Arabidopsis NRT1.6 and Embryo Development

Mesophyll protoplasts isolated from 3- to 4-week-old Arabidopsis leaves were transformed with column-purified DNA (Qiagen columns) by polyethylene glycol–mediated transformation (Sheen, 2001) and resuspended in WS medium (Sheen, 2001) containing 0.5 mM glucose and 0.2 mM KNO3. Expression of the fusion construct was monitored at various times after transformation by confocal laser scanning microscopy (LSM510; Zeiss) with excitation at 488 nm. The fluorescence emission signals were detected using a band-pass filter of 500 to 530 nm for GFP and a long-pass filter of 650 nm for the far-red autofluorescence of the chloroplast.

Phenotypic Analysis

The flowers and siliques of plants were minutely examined using a microscope (Z30; Olympus). Arrested seed development was noted by dissecting siliques from the primary shoot of self-pollinated plants. The wild-type and aborted seeds present in each silique were counted and expressed as the percentage of aborted seeds compared with the total number of seeds (aborted seeds plus well-developed seeds) in each silique. The length of each opened silique was also measured. At least 10 siliques were scored per plant. Three or four plants from each line were studied in each experiment, and the experiment was repeated at least three times.

To observe embryogenesis defects, siliques at 1 to 3 d after pollination from the wild type and mutants were dissected and the seeds cleared with Hoye’s solution (water/glycerol/chloral hydrate 3 mL/1 mL/8 mg) and observed on a Zeiss LSM510 META microscope equipped with differential interference contrast optics.

Measurement of the Nitrate Content of Arabidopsis Seeds

The nitrate content of seeds from the primary shoot of the wild type and nrt1.6-2 mutant was measured as described by Aboresi et al. (2005). Samples, each of 40 seeds, were homogenized in 500 μL 80% (v/v) ethanol at 4°C and incubated for 90 min. Nitrate content was determined by HPLC (Thayer and Huffaker, 1980) using a PARTISIL 10 SAX (strong anion exchange) column (Whatman) and 25 mM phosphate buffer, pH 2.8, as the mobile phase.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU266070 (NRT1.6 cDNA, At1g27080), At1g12110 (CHL1, NRT1.1), At1g69850 (NRT1.2), At2g26690 (NRT1.4), At1g32450 (NRT1.5), At1g08090 (NRT2.1), At1g08100 (NRT2.2), At1g12940 (NRT2.7), At3g54164 (PTR1), At2g02040 (PTR2), At4g05320 (UBQ10), At1g58360 (AAP1), At5g09220 (AAP2), At1g10010 (AAP8), At4g16370 (OPT3), At1g22710 (SUC2), At1g08100 (NRT2.1), and At5g22710 (SUC2).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Schematic Representation of the Gene Structure of NRT1.6.

Supplemental Figure 2. Amino Acid Sequence Alignment of the Arabidopsis Nitrate Transporters NRT1.1 (CHL1), NRT1.2, NRT1.3, NRT1.5, and NRT1.6.

Supplemental Figure 3. Abnormal Position of nrt1.6-2 Mutant Embryo.

Supplemental Figure 4. NRT1.6 Expression in the nrt1.6-1 Mutant.
Supplemental Figure 5. Quantitative PCR of AAP1 Expression.
Supplemental Data Set 1. Text File Corresponding to Supplemental Figure 2.

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REFERENCES


Characterization of the *Arabidopsis* Nitrate Transporter NRT1.6 Reveals a Role of Nitrate in Early Embryo Development

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