Two Phloem Nitrate Transporters, NRT1.11 and NRT1.12, Are Important for Redistributing Xylem-Borne Nitrate to Enhance Plant Growth[1][C][W][OPEN]

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This study of the Arabidopsis (*Arabidopsis thaliana*) nitrate transporters NRT1.11 and NRT1.12 reveals how the interplay between xylem and phloem transport of nitrate ensures optimal nitrate distribution in leaves for plant growth. Functional analysis in *Xenopus laevis* oocytes showed that both NRT1.11 and NRT1.12 are low-affinity nitrate transporters. Quantitative reverse transcription-polymerase chain reaction and immunoblot analysis showed higher expression of these two genes in larger expanded leaves. Green fluorescent protein and β-glucuronidase reporter analyses indicated that NRT1.11 and NRT1.12 are plasma membrane transporters expressed in the companion cells of the major vein. In *nrt1.11 nrt1.12* double mutants, more root-fed $^{15}NO_3^-$ was translocated to mature and larger expanded leaves but less to the youngest tissues, suggesting that NRT1.11 and NRT1.12 are required for transferring root-derived nitrate into phloem in the major veins of mature and larger expanded leaves for redistributing to the youngest tissues. Distinct from the wild type, *nrt1.11 nrt1.12* double mutants show no increase of plant growth at high nitrate supply. These data suggested that NRT1.11 and NRT1.12 are involved in xylem-to-phloem transfer for redistributing nitrate into developing leaves, and such nitrate redistribution is a critical step for optimal plant growth enhanced by increasing external nitrate.

Nitrate and ammonium are two major inorganic nitrogen sources for plants. In well-aerated soils, nitrification is rapid, and nitrate is the primary nitrogen source (Crawford and Forde, 2002). In addition to being an essential nutrient, nitrate also serves as a signaling molecule to break seed dormancy (Alboresi et al., 2005), induce leaf expansion (Walch-Liu et al., 2000), regulate lateral root development (Zhang and Forde, 2000), and coordinate the expression of nitrate-related genes (Ho et al., 2009). In order to utilize nitrate efficiently and regulate plant development precisely, nitrate uptake and allocation needs to be well modulated. Four gene families in Arabidopsis (*Arabidopsis thaliana*), NRT1/PTR (nitrate transporters/peptide transporters with 53 members), NRT2 (seven members), CLC (chloride channels with seven members), and SLAC1/SLAH (SLOW ANION CHANNEL-ASSOCIATED1 homologs with five members), have been identified and play diverse roles in nitrate transport of higher plants (Dechorgnat et al., 2011; Wang et al., 2012).

To acquire nitrate efficiently from soil under various conditions, plants have evolved two nitrate uptake systems: a low-affinity nitrate transport system and a high-affinity nitrate transport system. Three NRT2 transporters (NRT2.1, NRT2.2, and NRT2.4) and two NRT1 transporters (CHLORATE RESISTANCE1 [CHL1] and NRT1.2) are involved in nitrate uptake. While most of the NRT1 nitrate transporters are low-affinity nitrate transporters, CHL1 (NRT1.1) is distinct in that it is a dual-affinity nitrate transporter involved in both the low-affinity nitrate transport system and the high-affinity nitrate transport system (Tsay et al., 1993; Huang et al., 1996; Wang et al., 1998; Liu et al., 1999). Moreover, CHL1 also functions as a nitrate sensor responsible for the nitrate-regulated transcriptional response (Ho et al., 2009). Although there are four genes (NRT2.1, NRT2.2, NRT2.4, and CHL1) involved in the high-affinity nitrate transport system, each of them makes a unique contribution under certain conditions (Wang et al., 1998; Liu et al., 1999; Cerezo et al., 2001; Filleur et al., 2001; Orsel et al., 2002; Little et al., 2005; Li et al., 2007; Kiba et al., 2012). For example, NRT2.4 is induced by nitrogen starvation and makes a significant contribution at very low external nitrate concentration (Kiba et al., 2012).

Nitrate can be assimilated in root or shoot tissue. To transport nitrate to the aerial parts of the plant, nitrate has to be loaded into the xylem vessels in the root. NRT1.5 is responsible for loading nitrate into xylem for root-to-shoot nitrate transport (Lin et al., 2008). Moreover, root-to-shoot nitrate transport is negatively regulated by two

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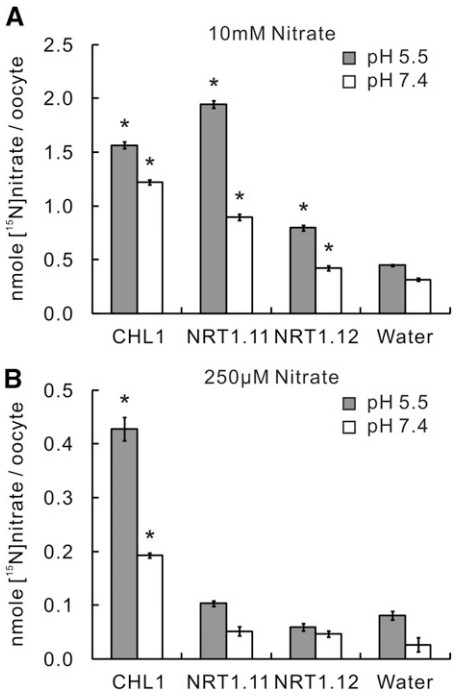

Figure 1. NRT1.11 and NRT1.12 exhibit low-affinity nitrate transport activity. Low-affinity (A) or high-affinity (B) nitrate uptake activity of *CHL1-*, *NRT1.11-*, *NRT1.12-*, or water-injected oocytes was examined by incubating oocytes with 10 mM or 250 μM $K^{15}NO_3$, respectively, at pH 5.5 or pH 7.4 for 1.5 h. ^{15}N retained in the oocytes was analyzed as described in "Materials and Methods." Values are means ± SE (n = 8–10 oocytes). Asterisks indicate significant differences ($P < 0.001$) compared with the water-injected control. Similar results were obtained using another two batches of oocytes.

low-affinity nitrate transporters, NRT1.8 and NRT1.9, expressed predominantly in the xylem parenchyma cells and the root companion cells, respectively (Li et al., 2010; Wang and Tsay, 2011). Nitrate transported to the shoot can be assimilated immediately into amino acids or stored in the vacuole via CLCa, a nitrate/proton exchanger located in the tonoplast (De Angeli et al., 2006).

Nitrate as well as organic nitrogen stored in a leaf can be remobilized to younger leaves during the vegetative stage and to seeds during the reproductive stage (Diaz et al., 2008; Fan et al., 2009). Depending on the plant species, nitrogen uptake may be partially or completely inhibited during the reproductive stage (Masclaux-Daubresse et al., 2010). Nitrogen remobilization from source leaves, therefore, is required to overcome the high nitrogen demand of seed filling. In addition, nitrogen deficiency will accelerate the senescence of source leaves to enhance nitrogen remobilization (Schulze et al., 1994; Guiboileau et al., 2012). During senescence, leaf protein undergoes proteolysis and the amino acids released are remobilized from source leaves to sink tissues (young leaves and seeds) via the phloem (Masclaux-Daubresse et al., 2010). In addition to amino acids, inorganic nitrogen in the form of nitrate can also be remobilized from source leaves. At the source sites, a low-affinity nitrate transporter, NRT1.7, expressed in the phloem of minor veins, is responsible for phloem loading of nitrate to allow nitrate to remobilize from older leaves to younger leaves (Fan et al., 2009). In addition, NRT2.4, a high-affinity nitrate transporter that is expressed in phloem

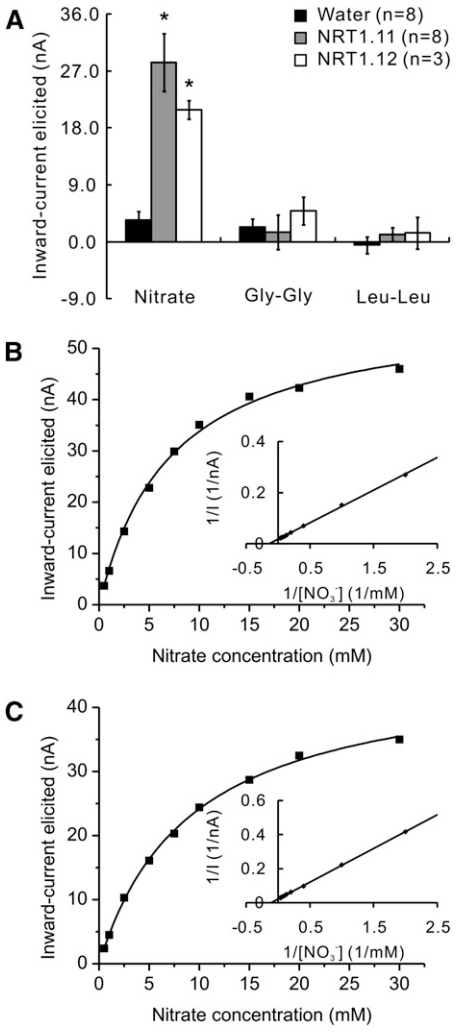

Figure 2. Substrate specificity and kinetic properties of NRT1.11 and NRT1.12 were analyzed by an electrophysiological approach. A, Substrate specificity of NRT1.11 and NRT1.12. Water-, *NRT1.11-*, or *NRT1.12*-injected oocytes were whole-cell voltage clamped at −60 mV. The inward currents elicited by 10 mM NO_3^-, 10 mM Gly-Gly, or 5 mM Leu-Leu at pH 5.5 were recorded. The currents shown are the differences between those in the presence and absence of the indicated substrate (nitrate or dipeptides). Values are means ± SE for the indicated number of oocytes recorded from three different frogs. Asterisks indicate significant differences ($P < 0.001$) compared with the water-injected control. B and C, Nitrate-elicited current in a single *NRT1.11*-injected (B) or *NRT1.12*-injected (C) oocyte. Oocytes were whole-cell voltage clamped at −60 mV. The inward currents elicited by different concentrations of NO_3^- at pH 5.5 were plotted as a function of the external nitrate concentration. In this particular experiment, the K_m was calculated by fitting to the Michaelis-Menten equation using a nonlinear least-squares method in the ORIGIN 5.0 program. The inset shows the Lineweaver-Burk plot. The r^2 value obtained using a linear fitting method in Microsoft Excel software is 0.999. Similar results were obtained from four oocytes from different frogs.

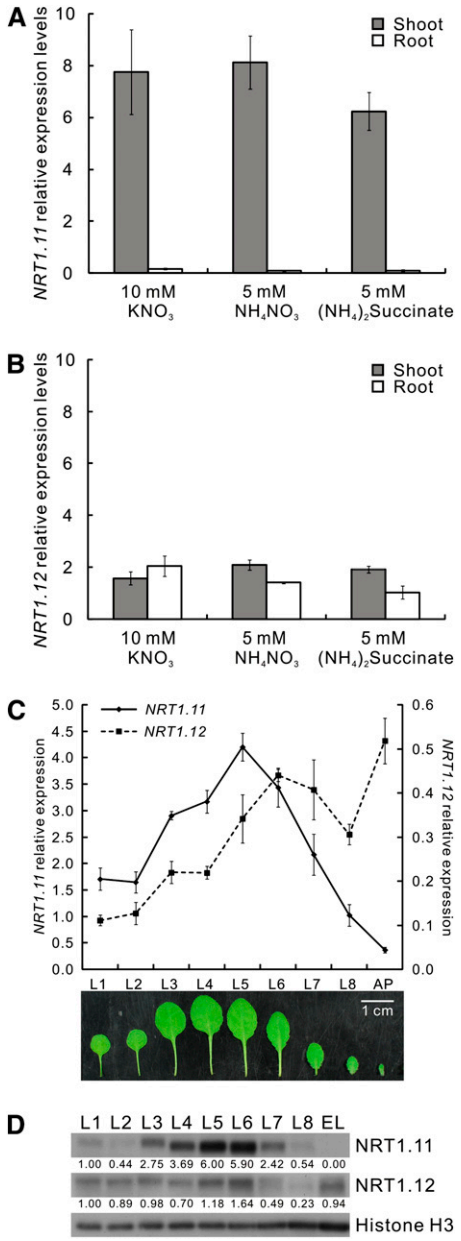

Figure 3. *NRT1.11* and *NRT1.12* show higher expression in larger expanded leaves. A and B, Quantitative RT-PCR analysis of *NRT1.11* and *NRT1.12* transcript levels in plants grown with three different nitrogen-supplemented media. Wild-type (Col-0) seedlings were grown for 9 d on a vertical plate containing different nitrogen concentrations as indicated. The relative amounts of *NRT1.11* (A) and *NRT1.12* (B) expression in roots and shoots were determined by quantitative RT-PCR using *UBQ10* as an internal control. Values are means ± SE for three biological repeats. C, Quantitative RT-PCR analysis of *NRT1.11* and *NRT1.12* transcript levels in different leaves. Wild-type (Col-0) seedlings were grown hydroponically with 1 mM NH_4NO_3 for 21 d. The relative amounts of *NRT1.11* and *NRT1.12* transcript levels in individual leaves were determined by quantitative RT-PCR using *UBQ10* as an internal control. Values are means ± SE for three plants. The bottom panel shows the representative sizes of individual leaves. D, Protein levels of NRT1.11 and NRT1.12 in different leaves. Wild-type (Col-0) seedlings were grown hydroponically with 1 mM NH_4NO_3 for 21 d. Total proteins from individual leaves were hybridized with anti-NRT1.11, anti-NRT1.12, or anti-histone H3 antibody as a loading control. The values of NRT1.11 and NRT1.12 protein levels normalized to histone H3, with the leaf 1 (the oldest true leaf) level set as 1, are indicated below the blots. In C and D, L1 to L8 indicate leaf numbers; EL indicates shoot apical meristem and emerging leaves. [See online article for color version of this figure.]

parenchyma cells and induced by nitrogen starvation, also affects the amount of nitrate loaded into the phloem for remobilization (Kiba et al., 2012). At the sink sites, to cope with nitrate supplied to the seed, a low-affinity nitrate transporter, NRT1.6, expressed in the funiculus is involved in delivering nitrate to developing seeds (Almagro et al., 2008). These studies suggested that nitrate and organic nitrogen stored in the source leaves can be remobilized via phloem to feed young tissues.

Xylem-to-phloem transfer, which transfers xylem-borne nutrients into the phloem, serves as an additional path to redistribute nitrogen into young tissues with low transpiration rates, including developing leaves and seeds (Marschner, 1995; Tegeder and Rentsch, 2010). After comparing the distribution of a xylem transport marker, [^{14}C]inulin carboxylic acid, and a phloem-permeable synthetic amino acid, [α-^{14}C]aminoisobutyric acid, in different leaves in tomato (*Solanum lycopersicum*), it was suggested that 5% of amino acids were imported to the top leaf via the xylem while 95% were imported via the phloem through xylem-to-phloem transfer in the stem (Van Bel, 1984). The contribution of xylem-to-phloem transfer to nitrogen import into developing leaves was further evidenced by the appearance of xylem-borne [^{14}C] Gln in the phloem of the upper stem and by blocking the transport of xylem-borne α-[^{14}C]aminoisobutyric acid into developing leaves by girdling in the stem or in the petiole of mature leaves below the developing leaves (Dickson et al., 1985; Da Silva and Shelp, 1990). At the molecular level, an amino acid permease, AAP2, expressed in the phloem was found to be involved in the xylem-to-phloem transfer of amino acids in source leaves and along the long-distance transport pathway (Zhang et al., 2010). A decrease of protein content and an increase of the carbon-nitrogen ratio in *aap2* seeds revealed the importance of xylem-to-phloem transfer in seed nutrition.

Little is known about whether xylem-to-phloem transfer also plays a critical function in the distribution of nitrate. In this study, we show that two NRT1 nitrate transporters, *NRT1.11* and *NRT1.12*, mediate the xylem-to-phloem transfer of nitrate and participate in nitrate redistribution from mature and larger expanded leaves to the youngest tissues. The phenotype of *nrt1.11 nrt1.12* double mutants indicates that this process is important for optimal plant growth in response to high nitrate.

RESULTS

NRT1.11 and NRT1.12 Are Low-Affinity Nitrate Transporters

NRT1.11 and NRT1.12, similar to NRT1.6, NRT1.7, and NRT1.9, are in subgroup IV of the Arabidopsis

NRT1/PTR family (Tsay et al., 2007). NRT1.11 and NRT1.12 share 78.7% amino acid sequence identity. Nitrate transporters characterized in the NRT1/PTR family are low-affinity nitrate transporters, except CHL1, which is a dual-affinity nitrate transporter. The high- and low-affinity nitrate uptake activities of NRT1.11 and NRT1.12 were assessed by analyzing $^{15}NO_3^-$ uptake activity of complementary RNA (cRNA)-injected oocytes at 10 mM and 250 μM, respectively. Compared with the water-injected control, *CHL1* cRNA-injected *Xenopus laevis* oocytes showed enhanced $^{15}NO_3^-$ uptake activities at both 10 mM and 250 μM, while *NRT1.11* and *NRT1.12* cRNA-injected oocytes only exhibited enhanced uptake at 10 mM but not at 250 μM (Fig. 1). As expected for proton-coupled transporters, 10 mM nitrate uptake activities of *NRT1.11* and *NRT1.12* cRNA-injected oocytes at pH 7.4 were 54% and 48% lower than those at pH 5.5, respectively (Fig. 1A). These results suggest that NRT1.11 and NRT1.12 are low-affinity nitrate transporters and that nitrate transport is pH dependent.

Nitrate transport activities of NRT1.11 and NRT1.12 were further confirmed by electrophysiological analysis of cRNA-injected oocytes. As shown in Figure 2A, inward currents were elicited by 10 mM nitrate in both *NRT1.11* and *NRT1.12* cRNA-injected oocytes, suggesting that they are electrogenic transporters. Some members of the NRT1/PTR family could transport dipeptides/tripeptides (Rentsch et al., 1995; Dietrich et al., 2004). To test whether NRT1.11 and NRT1.12 could transport dipeptides, electrophysiological analyses of cRNA-injected oocytes exposed to Gly-Gly and Leu-Leu were performed. No significant current differences were detected among *NRT1.11*-, *NRT1.12*-, and water-injected oocytes when incubated with Gly-Gly and Leu-Leu. These results suggest that NRT1.11 and NRT1.12 are nitrate transporters but not dipeptide transporters.

To determine the affinity of NRT1.11 and NRT1.12 for nitrate, current elicited by different concentrations of nitrate was recorded and calculated by fitting the Michaelis-Menten equation. The K_m values of NRT1.11 and NRT1.12 are approximately 7.2 and 9.2 mM, respectively (Fig. 2, B and C), confirming that NRT1.11 and NRT1.12 are low-affinity nitrate transporters.

NRT1.11 and *NRT1.12* Are Mainly Expressed in Larger Expanded Leaves

Expression levels of *NRT1.11* and *NRT1.12* in 9-d-old seedlings grown with different nitrogen sources, such as potassium nitrate, ammonium nitrate, or ammonium succinate, were investigated by quantitative reverse transcription (RT)-PCR analysis. As shown in Figure 3, A and B, *NRT1.11* was mainly expressed in the shoot, while *NRT1.12* was expressed in both shoot and root. In the shoot, the expression levels of *NRT1.11* were about 4-fold higher than in *NRT1.12*. The expression levels of these two genes showed no significant difference in three different nitrogen-supplemented media, suggesting that *NRT1.11* and *NRT1.12* expression is not affected by nitrogen sources.

Since the dominant gene, *NRT1.11*, is mainly expressed in shoot, the expression of these two genes in different leaves of 21-d-old plants was further analyzed. *NRT1.11* and *NRT1.12* expression was developmentally regulated, with the transcript level increasing from leaf 1 to leaf 5,

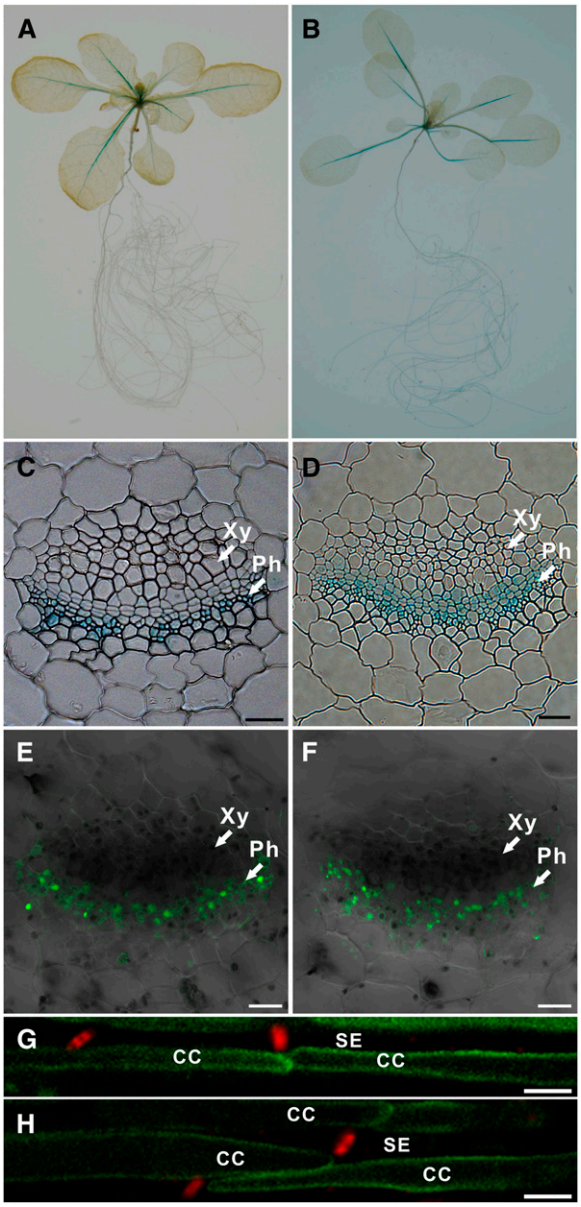

Figure 4. *NRT1.11* and *NRT1.12* are expressed in the companion cells of the major veins. A to D, Histochemical localization of GUS activity in a 21-d-old $NRT1.11_{pro}$:*GUS* plant (A and C) and a $NRT1.12_{pro}$:*GUS* plant (B and D) grown hydroponically with 1 mM NH_4NO_3. Images are whole plants (A and B) and cross sections of a petiole (C and D). E to H, Confocal images of $NRT1.11_{pro}$:*NRT1.11-GFP* (E and G) and $NRT1.12_{pro}$:*NRT1.12-GFP* (F and H) plants. Images are cross sections (E and F) and longitudinal sections of a petiole (G and H). Blue color in A to D shows GUS activity; green color in E to H shows GFP signal; red color in G and H is the aniline blue-stained sieve plate. CC, Companion cell; Ph, phloem; SE, sieve element; Xy, xylem. Bars = 20 μm in C to F and 5 μm in G and H.

reaching the maximal level in leaf 5 or 6, and then declining in younger leaves (Fig. 3C). The expression levels of NRT1.11 and NRT1.12 in different leaves were further confirmed by immunoblot analysis. Consistently, the NRT1.11 and NRT1.12 protein levels in 21-d-old plants were higher in leaf 5 and leaf 6 (Fig. 3D). These results suggest that *NRT1.11* and *NRT1.12* expression levels are more abundant in larger expanded leaves.

NRT1.11 and *NRT1.12* Are Expressed in the Companion Cells of Major Veins

To find out where *NRT1.11* and *NRT1.12* were expressed in actively growing leaves, the 1.6-kb upstream region of *NRT1.11* and *NRT1.12* was used to drive the expression of the *GUS* reporter gene and transformed into wild-type Columbia (Col-0) plants. In five independent transgenic lines for each gene, GUS staining in shoot was mainly found in major veins (a representative line is shown in Fig. 4, A and B). This tissue-specific expression pattern was identical in plants grown at different nitrate concentrations (Supplemental Fig. S1A). This expression pattern was further confirmed by quantitative RT-PCR analysis of the petiole, midrib, and lamina. As shown in Supplemental Figure S2, the expression levels in the petiole and the midrib were approximately 30 times higher for NRT1.11 and four times higher for NRT1.12 than their respective levels in lamina. After bolting, in addition to the major vein of rosette leaves, the expression of *NRT1.11* and *NRT1.12* could also be found in the major vein of cauline leaves, the junction of silique and pedicel, and the apex of silique (Supplemental Fig. S1B).

Cross sections of GUS-stained petiole revealed that GUS signals were restricted to the phloem side of the vascular tissues (Fig. 4, C and D). To confirm the expression of NRT1.11 and NRT1.12 in phloem, transgenic plants expressing fusion protein NRT1.11-GFP or NRT1.12-GFP driven by their native promoters were generated. As shown in Figure 4, E and F, consistent with the GUS staining pattern, NRT1.11-GFP and NRT1.12-GFP were located in the phloem of the major vein. In Figure 4, G and H, when sieve plates were revealed by aniline blue staining, the sieve elements can be recognized by the presence of sieve plates, and NRT1.11-GFP and NRT1.12-GFP signals were detected in the cells next to sieve elements and with similar end positions to the sieve elements, suggesting that NRT1.11 and NRT1.12 were expressed in companion cells.

NRT1.11 and NRT1.12 Are Localized in the Plasma Membrane

To investigate the subcellular localization of NRT1.11 and NRT1.12, NRT1.11-GFP and NRT1.12-GFP driven by the cauliflower mosaic virus 35S promoter were transiently expressed in Arabidopsis mesophyll protoplast. The NRT1.11-GFP and NRT1.12-GFP signals were found to be confined to a ring external to the chloroplasts (Fig. 5, A, B, D, and E), while the GFP control was

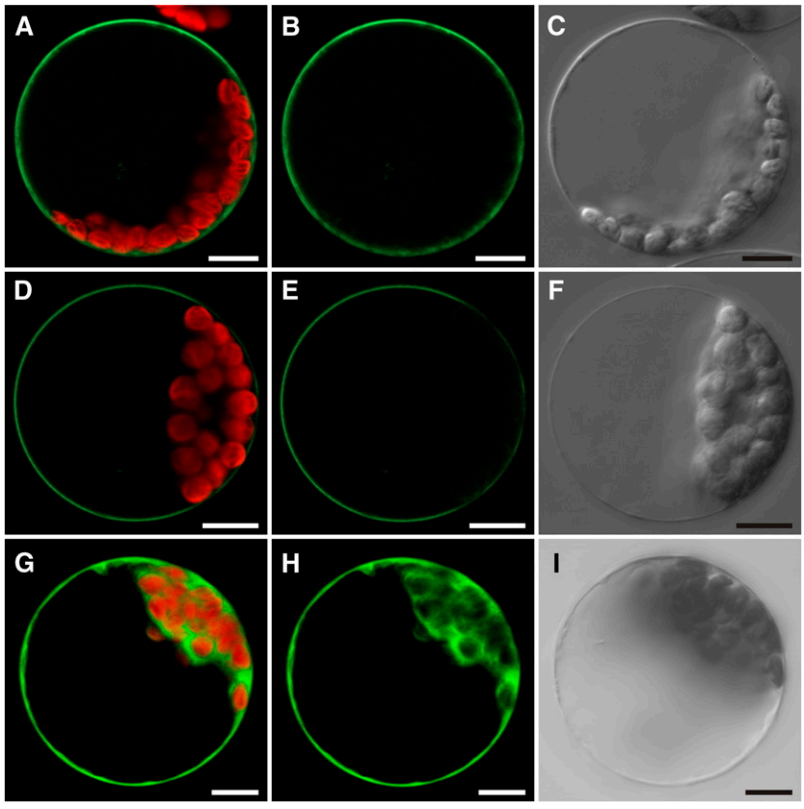

Figure 5. NRT1.11 and NRT1.12 are located in the plasma membrane. Subcellular localization is shown for NRT1.11-GFP fusion protein (A–C), NRT1.12-GFP fusion protein (D–F), or GFP alone (G–I) in Arabidopsis mesophyll protoplasts. *NRT1.11-GFP*, *NRT1.12-GFP*, or *GFP* was driven by the cauliflower mosaic virus 35S promoter and transiently expressed in Arabidopsis mesophyll protoplasts. Overlap images of GFP (green) and chlorophyll (red) fluorescence (A, D, and G), GFP fluorescence only (B, E, and H), and bright-field images (C, F, and I) are presented. Bars = 10 μm.

seen in the cytoplasm (Fig. 5, G and H). These results suggest that both NRT1.11 and NRT1.12 are localized in the plasma membrane.

NRT1.11 and NRT1.12 Facilitate Nitrate Redistribution from Mature and Larger Expanded Leaves to the Youngest Tissues

To determine the in vivo functions of *NRT1.11* and *NRT1.12*, two independent transfer DNA (T-DNA) insertion mutants in the Col-0 ecotype were isolated for each gene (Fig. 6A). In the *nrt1.11-1* and *nrt1.11-2* mutants, the T-DNA was inserted in the fourth exon and the second intron of *NRT1.11*, respectively. In the *nrt1.12-1* and *nrt1.12-2* mutants, the T-DNA was inserted in the second intron of *NRT1.12*. The T-DNA insertion was confirmed by genomic DNA PCR. Moreover, NRT1.11 or NRT1.12 protein could not be detected in the mutants by immunoblot analysis using NRT1.11- or NRT1.12-specific antibody (Fig. 6B), suggesting that they are null mutants. In addition, *nrt1.11-1 nrt1.12-1* and *nrt1.11-2 nrt1.12-2* double mutants were generated by crossing single mutants.

For the $^{15}NO_3^-$ allocation assay, $^{15}NO_3^-$ was fed to the root of wild-type (Col-0) and mutant plants for 30 min,

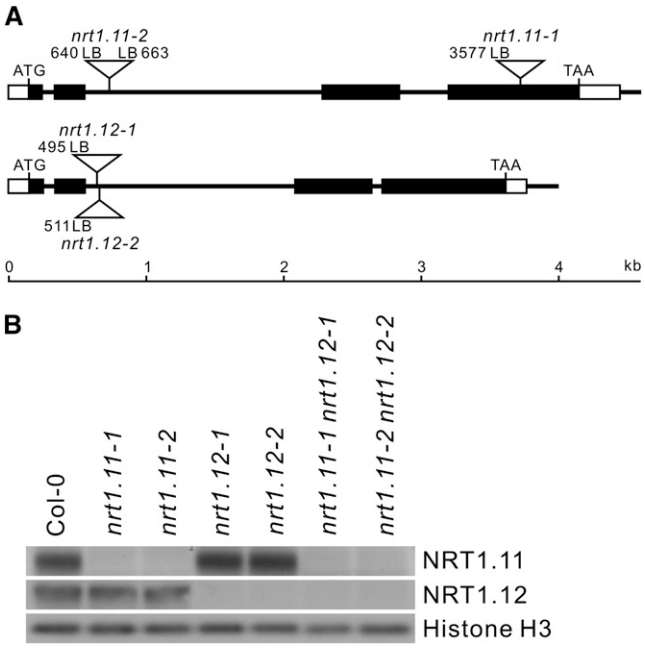

Figure 6. T-DNA-inserted mutants of *nrt1.11* and *nrt1.12* are null mutants. A, Schematic map of the T-DNA insertion sites in *nrt1.11-1*, *nrt1.11-2*, *nrt1.12-1*, and *nrt1.12-2* mutants. Black boxes and white boxes represent coding and untranslated regions, respectively. The numbers shown at the left border (LB) represent the positions of T-DNA insertion relative to the start codon (+1) in the genomic sequence. B, Immunoblot analysis of NRT1.11 and NRT1.12 protein in the wild type, *nrt1.11* and *nrt1.12*, and the *nrt1.11 nrt1.12* double mutant. Total protein isolated from the shoot of the wild type, *nrt.1.11-1*, *nrt1.11-2*, *nrt1.12-1*, *nrt1.12-2*, *nrt1.11-1 nrt12-1*, or *nrt1.11-2 nrt12-2* was used for immunoblot analysis. Histone H3 was used as a loading control.

and then the ^{15}N enrichment in individual leaves was analyzed. Compared with the wild type, no significant differences of the ^{15}N enrichment were found in *nrt1.11* and *nrt1.12* single mutants (Supplemental Fig. S3). However, as shown in Figure 7A, ^{15}N enrichment was higher in leaves 1 to 7 of *nrt1.11-1 nrt1.12-1* double mutants than in wild-type plants. Despite no statistical significance, ^{15}N enrichment in leaf 8 and the shoot apical meristem, including emerging leaves and leaf primordium, of *nrt1.11-1 nrt1.12-1* double mutants was slightly lower than in the wild type. To further elucidate the relative amount of ^{15}N transported to larger expanded leaves versus the youngest tissues, the ^{15}N enrichment ratios of leaf 8 and shoot apical meristem to leaf 6 were calculated and are shown in Figure 7B. In the *nrt1.11-1 nrt1.12-1* double mutant, the ratios were lower than in the wild type. Similar results were found in another double mutant, *nrt1.11-2 nrt1.12-2* (Fig. 7, C and D). When the data for the ^{15}N enrichment presented in Figure 7, A and C, were recalculated and converted, absolute value (μmol g^{-1} tissue; Supplemental Fig. S4, A and B) or relative ^{15}N distribution in different leaves (Supplemental Fig. S4, C and D) showed a similar pattern of higher values in the mature leaves of the double mutants but lower values in the younger tissues of the double mutants. The nitrate uptake activity and shoot-to-root ^{15}N ratio between the wild type and *nrt1.11 nrt1.12* double mutants showed no significant differences (Supplemental Fig. S5), suggesting that the increases of ^{15}N in the mature leaves are not due to the enhanced uptake or the increased nitrate transport to shoot in the *nrt1.11 nrt1.12* double mutant. These results supported the functional redundancy between *NRT1.11* and *NRT1.12* and showed that *NRT1.11* and *NRT1.12* work together to reduce the amount of nitrate transported to mature and larger expanded leaves but enhance the amount of nitrate allocated to the youngest tissues.

To further confirm the contribution of NRT1.11 and NRT1.12 in regulating nitrate allocation into the youngest leaf, nitrate content analysis was performed. In this experiment, residual nitrate in the leaves was first depleted by growing plants in the nitrate-free medium for 3 d. In addition, the nitrate reductase inhibitor tungstate was added to repress the rapid nitrate reduction in the leaf. As shown in Figure 8, nitrate concentration change in the youngest leaf of the *nrt1.11 nrt1.12* double mutant after shifting to nitrate-containing medium for 2 h was lower than in the wild type. This result together with the ^{15}N distribution experiments supported that NRT1.11 and NRT1.12 are involved in nitrate allocation to the youngest leaf.

The *nrt1.11 nrt1.12* Double Mutants Are Defective in High-Nitrate-Enhanced Growth

We next examined whether the loss of *NRT1.11* and *NRT1.12* has any effect on leaf growth by measuring the dry weights of individual true leaves in double

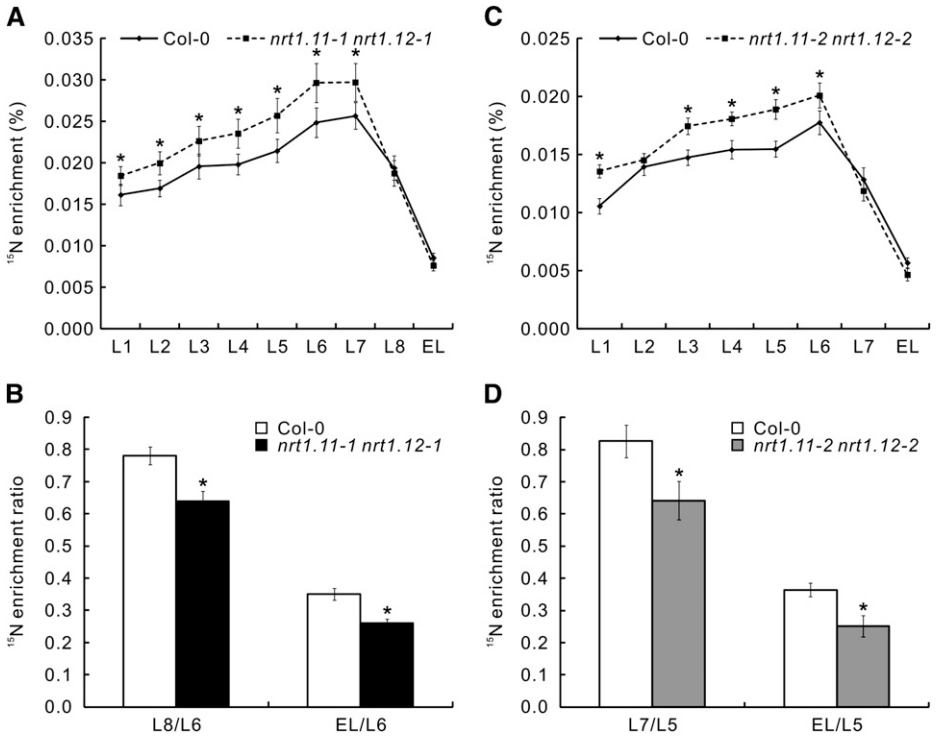

Figure 7. Double mutants *nrt1.11-1 nrt1.12-1* and *nrt1.11-2 nrt1.12-2* show altered leaf nitrate distribution. A and C, ^{15}N enrichment in the different leaves of the wild type and *nrt1.11 nrt1.12*. B and D, ^{15}N enrichment ratio of the youngest leaf and emerging leaves to larger expanded leaves in the wild type and *nrt1.11 nrt1.12*. The wild type, *nrt1.11-1 nrt1.12-1*, and *nrt1.11-2 nrt1.12-2* were grown hydroponically with 1 mM NH_4NO_3 for 21 d and then transferred to nutrient solution with 5 mM $K^{15}NO_3$ for 30 min. The ^{15}N enrichments of individual leaves presented are means ± SE from 11 independent plants (A and B) or nine independent plants (C and D). Asterisks indicate significant differences ($P < 0.05$) between the wild type and the double mutants. L1 to L8 are true leaf numbers, where leaf 1 is the oldest true leaf; EL represents emerging leaves and shoot apical meristem.

mutants. As shown in Figure 9A, increases of nitrate concentration from 0.2 mM to 1 or 5 mM were associated with increased growth in the wild type, particularly in younger leaves, leaf 4 and up. However, in *nrt1.11 nrt1.12* double mutants, no increase of the growth of younger leaves was observed when the nitrate concentrations were increased (Fig. 9, B and C). These results suggest that nitrate redistribution mediated by NRT1.11 and NRT1.12 is important for enhancing the growth of younger leaves at high nitrate concentrations.

DISCUSSION

NRT1.11 and NRT1.12 Affect the Nitrate Distribution in Leaves

Functional analysis in *X. laevis* oocytes showed that, similar to most nitrate transporters in the NRT1 (PTR) family, NRT1.11 and NRT1.12 are low-affinity nitrate transporters, with K_m of approximately 7.2 and 9.2 mM, respectively (Figs. 1 and 2). No dipeptide (Fig. 2A) or glucosinolate (Nour-Eldin et al., 2012) transport activity was detected in *NRT1.11*- and *NRT1.12*-injected oocytes. Recently, some members in subgroup I of the NRT1/PTR family were found to be able to transport abscisic acid (ABA), and ABA transport activity was restricted to a clade in subgroup I of NRT1/PTR (Kanno et al., 2012). Therefore, NRT1.11 and NRT1.12 in subgroup IV of NRT/PTR are less likely to transport ABA. Subcellular study showed that NRT1.11 and NRT1.12 are localized in the plasma membrane (Fig. 5). Taken together, these data suggested that NRT1.11 and NRT1.12 are plasma membrane nitrate transporters.

In the [^{15}N]nitrate feeding experiments, compared with the wild type, ^{15}N enrichments were higher in mature and larger expanded leaves (leaves 1–7) of *nrt1.11 nrt1.12* (Fig. 7, A and C), suggesting that

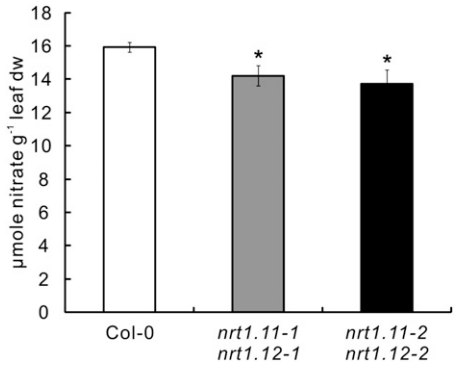

Figure 8. Nitrate allocated to the youngest leaf is reduced in *nrt1.11 nrt1.12* double mutants. The wild type and *nrt1.11-1 nrt1.12-1* were grown hydroponically with 1 mM NH_4NO_3 for 17 d, transferred to 1 mM NH_4Cl medium without nitrate and molybdate for 2 d, and then treated with 5 mM tungstate for 20 h. To measure the nitrate concentration changes in the youngest leaf, plants were further shifted to 10 mM KNO_3 with 5 mM tungstate for 2 h. The nitrate transported into the youngest leaf was calculated by subtracting the nitrate concentration in the plants before shifting to 10 mM KNO_3 medium. Data presented are means ± SE for eight biological replicates, each on a pool of leaves from three plants. A similar result was also observed in another independent experiment. Asterisks indicate significant differences ($P < 0.05$) between the wild type and the double mutants. dw, Dry weight.

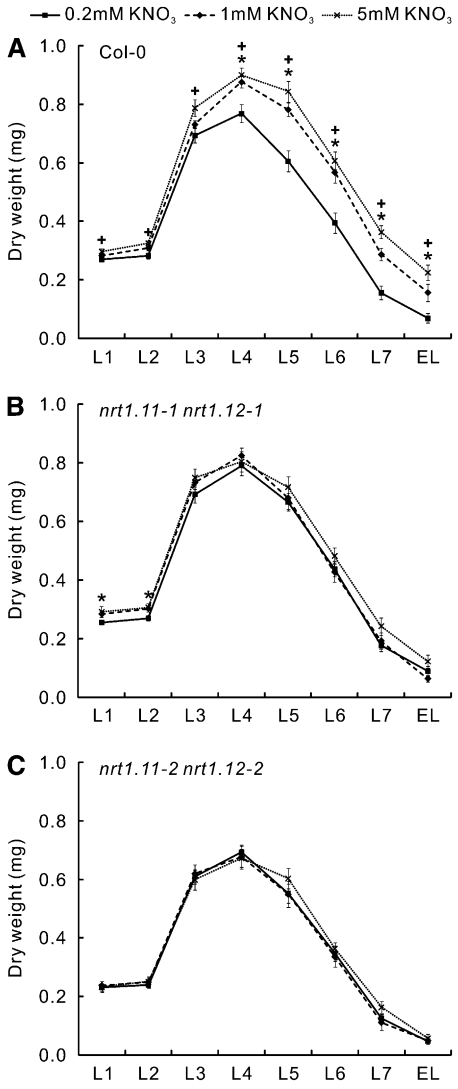

Figure 9. In *nrt1.11 nrt1.12* double mutants, a high concentration of nitrate did not enhance plant growth. Dry weight of individual leaves in the wild type (Col-0; A), *nrt1.11-1 nrt1.12-1* (B), and *nrt1.11-2 nrt1.12-2* (C) is shown. Plants were grown hydroponically with 0.2, 1, or 5 mM KNO_3 under a 16-h-light/8-h-dark cycle for 21 d. The leaf dry weights presented are means ± SE from 10 to 12 plants. Asterisks indicate significant differences ($P < 0.05$) between 0.2 and 1 mM KNO_3; crosses indicate significant differences ($P < 0.05$) between 0.2 and 5 mM KNO_3.

NRT1.11 and *NRT1.12* have inhibitory effects on the amount of nitrate translocated from the root into the mature and larger expanded leaves. *NRT1.11* and *NRT1.12* are highly expressed in the larger expanded leaves (leaves 3–7; Fig. 3, C and D). The GUS and GFP reporter assays showed that *NRT1.11* and *NRT1.12* are expressed in the companion cells of the major veins (Fig. 4). These data suggest that in the major vein, NRT1.11 and NRT1.12 could load nitrate into the phloem, and this process will reduce the amount of nitrate remaining in the xylem to be delivered to the mature and larger expanded leaves. Nitrate allocated to the phloem should follow the source-to-sink direction to be transported to the youngest leaves or root. Indeed, the ^{15}N enrichment ratio of the youngest leaves to the larger expanded leaves (Fig. 7, B and D) as well as the nitrate allocation to the youngest leaf in the *nrt1.11 nrt1.12* double mutant (Fig. 8) were lower than in the wild type.

The distribution of a root-derived nutrient in the shoot mainly depends on the transpiration stream of xylem but can be modified by the interaction between xylem and phloem. Xylem-to-phloem transfer is important for transporting root-derived amino acids, boron, iron, and rubidium into young leaves or reproductive tissues with low transpiration activities (Sharkey and Pate, 1975; McNeil et al., 1979; Dickson et al., 1985; Feller, 1989; Da Silva and Shelp, 1990; Shelp et al., 1998; Tsukamoto et al., 2009). Nevertheless, some nutrients like strontium and calcium are transported restrictively in the xylem, and strontium is usually used as a xylem transport marker (Feller, 1989; Shelp et al., 1998). In the past, it was not known if xylem-to-phloem transfer was involved in transporting xylem-borne nitrate to young developing tissues. Our study of *NRT1.11* and *NRT1.12* provides evidence for the presence of xylem-to-phloem transfer in redistributing xylem-derived nitrate into developing tissues.

Functional Comparison of NRT1.11/NRT1.12 with Other Phloem Transporters

In general, nitrate concentration in phloem is much lower than that of amino acid and ammonium (Peuke and Jeschke, 1993). Nitrogen composition in phloem might be altered at different parts of the plant or affected by external nitrogen supplied. For example, in the cotyledon of *Ricinus communis*, only nitrate but not ammonium can be loaded into the phloem (Schobert and Komor, 1992). Or, when increasing external nitrate concentration from 0.2 to 4 mM, it was found that phloem nitrate concentration can increase dramatically from 0.06 to 1.47 mM (Peuke et al., 1996). Although nitrate is not the major form of nitrogen in phloem, several recent studies of NRT1 transporters show that phloem nitrate transport plays critical roles in nitrate allocation (Wang et al., 2012).

It is interesting that all of the four phloem-localized nitrate transporters, NRT1.6, NRT1.7, NRT1.11, and NRT1.12, belong to subgroup IV of the NRT1/PTR family. Similar to *NRT1.11* and *NRT1.12*, *NRT1.7* is also expressed in phloem and important for leaf nitrate distribution (Fan et al., 2009). However, there are differences between them: (1) *NRT1.11* and *NRT1.12* are expressed in the major vein, whereas *NRT1.7* is expressed in the minor vein; and (2) *NRT1.11* and *NRT1.12* are expressed in larger expanded leaves, showing maximal levels in leaves 5 and 6 (Fig. 3, C and D), whereas *NRT1.7* is expressed in older leaves, showing maximal levels in leaves 1 and 2 (Fan et al., 2009). Therefore, NRT1.11 and NRT1.12 expressed in the major vein are involved in

loading xylem-borne nitrate into the phloem for redistribution, while NRT1.7 expressed in the minor vein is involved in remobilizing nitrate stored in the mesophyll of older leaves. In the *nrt1.7* mutant, nitrate concentrations in the phloem exudates collected from older leaves are reduced (Fan et al., 2009). However, as expected for NRT1.11 and NRT1.12 being responsible for transferring xylem-borne nitrate to phloem in the petiole, nitrate concentrations in the phloem exudates collected from detached leaf with no nitrate source from xylem show no difference between the wild type and double mutants (Supplemental Fig. S6). Although NRT1.11/NRT1.12 and NRT1.7 all participate in allocating nitrate into developing tissue via the phloem, they remobilize nitrate from different sources using distinct spatial and temporal expression patterns. By working together, they could guarantee sufficient nitrate supply into demanding tissues.

Similar to NRT1.11 and NRT1.12, amino acid transporter AAP2, which is also expressed in the phloem of the leaf major vein, is responsible for xylem-to-phloem transfer of xylem-borne amino acids to fed sink tissues, suggesting that the major vein is the primary site of the xylem-to-phloem transfer in the leaf (Zhang et al., 2010). Compared with *NRT1.11* and *NRT1.12*, *AAP2* was mainly expressed in much older leaves, suggesting that xylem-to-phloem transfer of organic nitrogen (amino acids) and inorganic nitrogen (nitrate) occurred in the leaves at different developmental stages (Supplemental Fig. S7).

Xylem-to-phloem transfer serves as a critical pathway for the redistribution of nutrients into sink tissues. In addition to the leaf major vein, xylem-to-phloem transfer can also take place in the nodal region and root. A boron transporter, NIP6;1 (for NOD26-like intrinsic protein6;1), is involved in xylem-to-phloem transfer of boron in nodal regions to fed reproductive tissues (Tanaka et al., 2008). A nitrate transporter, NRT1.9, expressed in root phloem is involved in transferring xylem nitrate into phloem to regulate root-to-shoot nitrate transport (Wang and Tsay, 2011). Studies of NRT1.11/NRT1.12 and NRT1.9 indicate that plants use different nitrate transporters to regulate xylem-to-phloem transfer at different parts of the plant.

NRT1.11 and NRT1.12 Are Important for High-Nitrate-Enhanced Leaf Growth

Nitrogen availability is a key factor in biomass production and crop yield. Indeed, in the wild type, increase in nitrate concentration from 0.2 mM to 1 or 5 mM was associated with increased biomass, predominantly in younger leaves (Fig. 9A). In response to high nitrate, no such increase of leaf growth was found in *nrt1.11 nrt1.12* double mutants (Fig. 9, B and C), indicating that xylem-to-phloem transfer mediated by NRT1.11 and NRT1.12 for nitrate redistribution in leaves is important for high-nitrate-enhanced shoot growth. The amino acid concentrations in the phloem sap can be 30 to 100 times higher than that of nitrate (Allen and Smith, 1986; Hayashi and Chino, 1986; Shelp, 1987; Peuke, 2010). Although nitrate is not the major nitrogen in the phloem, the growth phenotype of *nrt1.11 nrt1.12* double mutants suggested that phloem-mediated nitrate redistribution is not dispensable, and compared with amino acid, nitrate may have additional effects on young leaf growth as a signaling molecule, osmoticum, and/or regulator of redox stasis. Several transporters in the NRT1/PTR family are found to be involved in hormone transport (Krouk et al., 2010; Kanno et al., 2012). Although the possibility of hormone involved in the growth phenotype of *nrt1.11 nrt1.12* double mutants cannot be completely eliminated, nitrate-dependent differences of the growth phenotype as well as the nitrate transport activity of NRT1.11 and NRT1.12 suggest that altered nitrate distribution plays a role for the growth phenotype. This growth phenotype suggests that, in addition to nitrogen uptake and assimilation, leaf nitrate redistribution is another potential key step in modulating plant nitrogen use efficiency. It will be interesting to see if an NRT1.11/NRT1.12-associated nitrate redistribution mechanism is responsible for the distinct nitrogen use efficiency behavior of different crop species or varieties.

As mentioned above, NRT1.7 is responsible for remobilizing stored nitrate in older leaves, while NRT1.11/NRT1.12 are responsible for redistributing xylem-borne nitrate in larger expanded leaves. Compared with the wild type, *nrt1.7* showed growth retardation at low nitrate conditions, while *nrt1.11 nrt1.12* double mutants show growth defects at high nitrate conditions. This indicated that plants use different strategies of nitrate allocation to cope with different nitrate conditions to sustain vigorous growth. Study of *NRT1.11/NRT1.12* will provide additional tools to manipulate and understand the relationship between nitrate redistribution and plant growth.

MATERIALS AND METHODS

Functional Expression of NRT1.11 and NRT1.12 in *Xenopus laevis* Oocytes

NRT1.11 or *NRT1.12* cDNA was amplified by PCR using primer pairs *NRT1.11* forward (5′-cccggatccATGGAGAACCCTCCCAATGAAAC-3′) with *Bam*HI site and *NRT1.11* reverse (5′-ccccccgggCAAGCTTGCAAGACCTATTGTTCC-3′) with *Xma*I site or *NRT1.12* forward (5′-cccggatccATGGAGACCCTCCGGA-3′) with *Bam*HI site and *NRT1.12* reverse (5′-cccggatccCCTAAGAAAATCCAAATCCATGA-3′) with *Bam*HI site, respectively. Lowercase letters indicate nonspecific nucleotides, and underlined letters indicate where restriction sites were created. PCR products were cloned into pGEM-T Easy vector (Promega). After sequencing, *NRT1.11* and *NRT1.12* cDNAs were subcloned into the *Eco*RI and *Bam*HI sites of the oocyte expression vector pGEM-HE (Liman et al., 1992), respectively. The pGEM-HE *NRT1.11* and pGEM-HE *NRT1.12* plasmids were linearized using *Nhe*I, and capped mRNA was transcribed in vitro by using mMESSAGE mMACHINE kits (Ambion). Oocytes were isolated and injected with 100 ng of *NRT1.11* or *NRT1.12* cRNA in 50 nL of water, as described previously (Tsay et al., 1993), except that the Barth solution was replaced with ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4). After 2 d of incubation in the ND96 solution containing 0.005% (w/v) gentamycin, nitrate uptake assays using [^{15}N]nitrate were performed as described previously using a continuous-flow isotope ratio mass spectrometer coupled with a carbon nitrogen elemental analyzer (ANCA-GSL MS; PDZ Europa; Almagro et al., 2008; Lin et al., 2008). *CHL1* cRNA and water-injected oocytes were used as

positive and negative controls, respectively. Electrophysiological analyses of injected oocytes were performed as described previously (Huang et al., 1999), except that the $CsNO_3$ was replaced with HNO_3.

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used as the wild-type control. The Arabidopsis T-DNA mutants *nrt1.11-1* (SALK_099498), *nrt1.11-2* (SALK_065633), *nrt1.12-1* (SALK_013091), and *nrt1.12-2* (SAIL_270_A08) were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/; Sessions et al., 2002; Alonso et al., 2003). The T-DNA insertions were confirmed by genomic PCR using left border primers LBa1 (5′-TCAAACAGGATTTTCGCCTGC-3′) for SALK lines or LB3 (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′) for the SAIL line and paired with gene-specific primers as follows: *nrt1.11-1* (forward, 5′-TCTGAAATCAAAGCTCCAAGTCAAAA-3′; reverse, 5′-ACTTAATAACACCACACACCAATGCAATA-5′); *nrt1.11-2* (forward, 5′-ACTTCCTCTTCTTCTTCCTCTTCCTTTTT-3′; reverse, 5′-AAAACCTCGTACCACCATCCTTCA-3′); *nrt1.12-1* and *nrt1.12-2* (forward, 5′-ATGGAGAACCCTCCGGATCA-3′; reverse, 5′-CTGTAGATGTCTTCCAAAAAAAG-3′). The *nrt1.11-1 nrt1.12-1* and *nrt1.11-2 nrt1.12-2* double mutants were generated by crossing the indicated single mutants. For analyzing gene expression in three different nitrogen-containing media, Col-0 plants were grown under continuous light conditions on a vertical plate containing 10 mM KNO_3, 5 mM NH_4NO_3, or 5 mM $(NH_4)_2$-succinate with 10 mM KH_2PO_4/K_2HPO_4, basal nutrient (2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM $FeSO_4$-EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4 \cdot 2H_2O$, 1 μM $ZnCl_2$, 1 μM $CuSO_4 \cdot 5H_2O$, and 0.2 μM $NaMoO_4 \cdot 2H_2O$), 0.1% (w/v) MES, 0.5% (w/v) Suc, and 0.45% (w/v) agarose, at pH 5.5 (adjusted with KOH). The nutrient solutions used for hydroponic cultivation contained different concentrations of KNO_3 or NH_4NO_3 with 1 mM KH_2PO_4/K_2HPO_4, the basal nutrient indicated above, and 0.1% (w/v) MES, at pH 5.5 (adjusted with KOH). Seeds were germinated on rockwool in one-tenth-diluted nutrient solution under long-day conditions (16 h of light/8 h of dark, 65 μmol m^{-2} s^{-1} photosynthetic photon flux density) at 23°C using an Araponics growing system. After 3 d, full-strength nutrient solution was applied and renewed twice per week. The light intensity was measured using an LI-250A light meter with an LI-190SA quantum sensor (LI-COR).

Quantitative RT-PCR

Total RNA was extracted from roots, shoots, or different leaves using TRIzol reagent (Gibco BRL). The first-strand cDNAs were synthesized using oligo(dT) primers and ImProm-II reverse transcriptase (Promega). Primers specific for the *NRT1.11*, *NRT1.12*, or *UBQ10* gene were designed by Primer Express 2.0 software (Applied Biosystems). Quantitative PCR was performed with the ABI PRISM 7500 sequence detection system (Applied Biosystems) programmed for 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers used were as follows: *NRT1.11* (forward, 5′-TACTCGCGATAATGAGCTTCATCA-3′; reverse, 5′-CTCTCTCACACCATTAACCCTACCA-3′), *NRT1.12* (forward, 5′-TATGCACAACGGACCAAGTTGA-3′; reverse, 5′-GAGTTCTGGCTAACGTTTATCGACA-3′), and *UBQ10* (forward, 5′-AGAAGTTCAATGTTTCGTTTCATGTAA-3′; reverse, 5′-GAACGGAAACATAGTAGAACACTTATTCA-3′). The relative amount of gene expression was determined by the comparative threshold cycle (C_T) method using *UBQ10* as an internal control. The mean values were averages of $2^{-\Delta C_T} \times 100$ ($\Delta C_T = C_{T,\text{ gene of interest}} - C_{T,\text{ UBQ10}}$) from three biological repeats.

Antibodies and Immunoblots

The anti-NRT1.11 and anti-NRT1.12 rabbit polyclonal antibodies were generated and purified using synthetic peptide NETEAKQIQTNEGKKTKC for NRT1.11 or DQTESKETLQQPITRRRTKC for NRT1.12 by LTK BioLaboratories (http://www.ltk.com.tw). Underlined amino acids are specific epitopes corresponding to residues 6 to 22 for NRT1.11 and residues 6 to 24 for NRT1.12. The Cys residue at the C terminus was added to facilitate antibody purification.

To isolate total protein, tissues were homogenized in ice-cold extraction buffer (300 mM Tris-HCl, pH 8.0, 8% SDS, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was then centrifuged at 10,000*g* for 15 min, and the supernatant, the total protein, was collected in a chilled tube. The concentration of total protein was determined by the Pierce BCA Protein Assay Kit (Thermo Scientific). For immunoblot analysis, 30 μg of protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Detections were performed using the Amersham ECL Western Blotting Detection Reagents (GE Healthcare). Anti-NRT1.11, anti-NRT1.12, anti-histone H3 (ab1791; Abcam), and horseradish peroxidase-linked anti-rabbit IgG antibody (NA934; GE Healthcare) were used at dilutions of 1:1,000, 1:1,000, 1:2,000, and 1:5,000, respectively. Signals were detected by Amersham Hyperfilm ECL (GE Healthcare) and quantified by ImageJ software (National Institutes of Health).

Histochemical Analysis of Promoter Activity

A 1.56-kb genomic fragment of the *NRT1.11* promoter (−1,566 to −1 bp) or a 1.59-kb genomic fragment of the *NRT1.12* promoter (−1,593 to −1 bp) was generated by PCR using the primers *NRT1.11* forward (5′-aagtccATACAGTTAATCGACAATGT-3′) with *Hin*dIII site and *NRT1.11* reverse (5′-ggatccTGTTGAATGTGATTTGATCA-3′) with *Bam*HI site or *NRT1.12* forward (5′-ggatccAGCCTGTAGGATATAGAATCACAAA-3′) with *Bam*HI site and *NRT1.12* reverse (5′-ggatccTGTTGTTACCTTTCAAGCTTCGA-3′) with *Bam*HI site. Lowercase letters indicate nonspecific nucleotides, and underlined letters indicate where restriction sites were created. After sequencing, the fragments were cloned in front of the *uidA* (*GUS*) gene in the binary vector pBI101.1. The binary vectors were introduced into wild-type (Col-0) plants using the *Agrobacterium tumefaciens* (strain GV3101)-mediated floral dip method (Clough and Bent, 1998). Putative transformants were selected on one-half-strength Murashige and Skoog plates containing 0.005% (w/v) kanamycin. GUS staining was performed using the protocol described by Lagarde et al. (1996) with some modification. The 21-d-old homozygous transgenic plants (T3), cultivated hydroponically with 1 mM NH_4NO_3 as described previously, were vacuum infiltrated in prefix solution (0.5% [v/v] formaldehyde, 0.05% [v/v] Triton X-100, and 50 mM sodium phosphate, pH 7.0) and incubated in prefix solution at room temperature for 1.5 h. After three rinses with 50 mM sodium phosphate, pH 7.0, the $NRT1.11_{pro}$-*GUS* transgenic plants and the $NRT1.12_{pro}$-*GUS* transgenic plants were incubated for 2.5 and 4.5 h, respectively, at 37°C in X-Gluc staining solution (50 mM sodium phosphate, pH 7.0, 0.05% [v/v] Triton X-100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, and 1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide). After three washes with 50 mM sodium phosphate, pH 7.0, the seedlings were fixed in 2% (v/v) formaldehyde, 0.5% (v/v) glutaraldehyde, and 100 mM sodium phosphate, pH 7.0, at room temperature overnight. Tissues were cleared through a graded ethanol serious in order to remove pigments. The whole-seedling images were taken by a Nikon D80 digital camera with a Sigma 18- to 50-mm F2.8 EX DC Macro lens. For sections, the stained petioles were further dehydrated in a graded series of ethanol washes and embedded in LR White medium-grade acrylic resin (London Resin). Then, 6-μm semifine sections were cut, mounted on glass slides, and visualized on an AxioImager-Z1 (Zeiss).

NRT1.11-GFP and NRT1.12-GFP Localization in Transgenic Plants

A 6.06-kb genomic fragment of *NRT1.11*, including the 2.06-kb upstream region of the translation start site and 4.00 kb of the coding region, or a 5.48-kb genomic fragment of *NRT1.12*, including the 2.02-kb upstream region of the translation start site and 3.48 kb of the coding region, was amplified from wild-type (Col-0) genomic DNA by PCR using the *NRT1.11* forward primer 5′-ggtaccGAGCTATGCATTTTCTTGTGATATT-3′ with *Kpn*I site and the *NRT1.11* reverse primer 5′-ggtaccATTGGTTTTAACAACTGGACTTAGAT-3′ with *Kpn*I site or the *NRT1.12* forward primer 5′-ggtaccCATCATTATGGCTTTTTATTCACTT-3′ with *Kpn*I site and the *NRT1.12* reverse primer 5′-ggtaccGTTTAATTTAATAACTTCCTCTTCTTCTTT-3′ with *Kpn*I site. Lowercase letters indicate nonspecific nucleotides, and underlined letters indicate where restriction sites were created. After sequencing, the $NRT1.11_{pro}$:*NRT1.11-GFP* fragment or the $NRT1.12_{pro}$:*NRT1.12-GFP* fragment was cloned in frame with *GFP* in the binary vector pMDC107 (Curtis and Grossniklaus, 2003) at the *Kpn*I site in the right orientation. The pMDC107 $NRT1.11_{pro}$:*NRT1.11-GFP* plasmids and the pMDC107 $NRT1.12_{pro}$:*NRT1.12-GFP* plasmids were introduced into *nrt1.11-1* and *nrt1.12-1* mutant plants, respectively, using the *A. tumefaciens* (strain GV3101)-mediated floral dip method (Clough and Bent, 1998). Putative transformants were selected on one-half-strength Murashige and Skoog plates containing 0.0025% (w/v) hygromycin B using the methods described previously (Harrison et al., 2006). For NRT1.11-GFP and NRT1.12-GFP protein observation, the leaf 5 or 6 petioles of 21-d-old plants grown hydroponically with 1 mM NH_4NO_3 as described previously were embedded in 5% (w/v) agarose dissolved in 1× phosphate-buffered saline and cut into 120-μm sections with a Vibratome Series

1000 (Technical Products International). Sieve plates were stained with 0.05% aniline blue in 1× phosphate-buffered saline buffer for 5 to 10 min. The slices of agarose were mounted on slides before observation using a confocal Zeiss LSM780 microscope. Green fluorescence was visualized with excitation at 488 nm and a band-pass filter of 480 to 540 nm. Aniline blue fluorescence was visualized with excitation at 405 nm and a band-pass filter of 490 to 570 nm.

Subcellular Localization by Arabidopsis Protoplast Transformation

NRT1.11 or *NRT1.12* cDNA was amplified by PCR using the *NRT1.11* forward primer 5′-ggatccATGGAGAACCCTCCCAA-3′ with *Bam*HI site and the *NRT1.11* reverse primer 5′-ggatccAATTGGTTTTAACAACTGGA-3′ with *Bam*HI site or the *NRT1.12* forward primer 5′-ggatccATGGAGAACCCTC-CGGATCA-3′ with *Bam*HI site and the *NRT1.12* reverse primer 5′-ggatcc-AGTTTAATTTAATAACTTCCTCT-3′ with *Bam*HI site. Lowercase letters indicate nonspecific nucleotides, and underlined letters indicate where restriction sites were created. The amplified fragments were then cloned in frame with *GFP* in the vector 326-GFP (Lee et al., 2001) at the *Bam*HI site, resulting in the *NRT1.11-GFP* or *NRT1.12-GFP* construct under the control of the 35S promoter. The fusion construct or the vector 326-GFP was then transiently expressed in Arabidopsis protoplasts. Arabidopsis protoplast transformation was performed following the protocol described by Sheen (2001). Arabidopsis protoplasts were isolated from leaf tissues of 3- to 4-week-old plants grown on soil. GFP-fused plasmids isolated by the Qiagen plasmid kit were transformed into protoplasts. After incubation in W5 solution under light for 16 to 24 h, fluorescent cells were imaged as described by Wang and Tsay (2011).

$^{15}NO_3^-$ Labeling Assay

Plants were grown hydroponically with 1 mM NH_4NO_3 for 21 d. At 8 h into the photoperiod, plants were transferred to 5 mM KNO_3 hydroponic medium containing a 9.8% excess of ^{15}N for 30 min, and then individual leaves were collected. ^{15}N abundance in each leaf was analyzed as described previously using a continuous-flow isotope ratio mass spectrometer coupled with a carbon nitrogen elemental analyzer (ANCA-GSL MS; PDZ Europa; Fan et al., 2009). The ^{15}N abundance was calculated as atom percent and defined as $(^{15}N)/(^{15}N + {}^{14}N) \times 100\%$. The ^{15}N enrichment was calculated as ^{15}N abundance in labeled leaf minus ^{15}N abundance in nonlabeled leaf.

Nitrate Content Analysis by HPLC

Leaves were frozen in liquid N_2 and dried by lyophilization. After measuring the dry weight, nitrate in the leaves was extracted by boiling in distilled water for 30 min. Nitrate concentration was then determined by HPLC (Thayer and Huffaker, 1980) using a PARTISIL 10 strong anion exchanger column (Whatman), with 50 mM KH_2PO_4 buffer, pH 3.0, as the mobile phase.

Collection and Analysis of Phloem Exudates

Leaf phloem exudates were collected from excised leaves using procedures modified from a protocol described previously (Deeken et al., 2008). Cut leaves were immediately recut at the tip of the petiole under exudate buffer (2.5 mM Na_2EDTA, pH 7.5, osmotically adjusted to 230 mosmol with sorbitol) with fresh razor blades without wounding. The cut leaves were rinsed once in the fresh exudation buffer to remove contaminants and then placed in 200 μL of new exudate buffer. During phloem sap exudation, the leaves were illuminated (30 μE) and incubated in a humid and CO_2-saturated box. After 2 h of bleeding, the buffer solution containing phloem exudates was analyzed for nitrate and sugar content as described (Wang and Tsay, 2011).

The Arabidopsis Genome Initiative numbers for the genes mentioned in this article are as follows: *NRT1.11* (At1g52190), *NRT1.12* (At3g16180), *CHL1* (At1g12110), *NRT1.2* (At1g69850), *NRT1.5* (At1g32450), *NRT1.6* (At1g27080), *NRT1.7* (At1g69870), *NRT1.8* (At4g21680), *NRT1.9* (At1g18880), *NRT2.1* (At1g08090), *NRT2.2* (At1g08100), *NRT2.4* (At5g60770), *SLAC1* (At1g12480), *CLCa* (At5g40890), *AAP2* (At5g09220), *NIP6;1* (At1g80760), and *UBQ10* (At4g05320).

Supplemental Data

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

Supplemental Figure S1. Tissue-specific expression patterns of *NRT1.11* and *NRT1.12*.

Supplemental Figure S2. *NRT1.11* and *NRT1.12* expression is higher in petiole and midrib.

Supplemental Figure S3. No difference in leaf nitrate distribution is observed in *nrt1.11* and *nrt1.12* single mutants.

Supplemental Figure S4. ^{15}N concentration and ^{15}N distribution in different leaves of the wild type and *nrt1.11 nrt1.12* double mutants.

Supplemental Figure S5. Nitrate uptake activity and shoot-to-root ^{15}N ratios in the wild type and *nrt1.11 nrt1.12* double mutants.

Supplemental Figure S6. Nitrate concentrations of leaf phloem exudates in the wild type and *nrt1.11 nrt1.12* double mutants.

Supplemental Figure S7. Relative expression levels of *AAP2*, *NRT1.11*, and *NRT1.12* in different leaves.

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