Arabidopsis NRT1.5 Is Another Essential Component in the Regulation of Nitrate Reallocation and Stress Tolerance[1][W]

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Nitrate reallocation to plant roots occurs frequently under adverse conditions and was recently characterized to be actively regulated by Nitrate Transporter1.8 (NRT1.8) in Arabidopsis (*Arabidopsis thaliana*) and implicated as a common response to stresses. However, the underlying mechanisms remain largely to be determined. In this study, characterization of NRT1.5, a xylem nitrate-loading transporter, showed that the mRNA level of *NRT1.5* is down-regulated by salt, drought, and cadmium treatments. Functional disruption of *NRT1.5* enhanced tolerance to salt, drought, and cadmium stresses. Further analyses showed that nitrate, as well as Na^+ and Cd^{2+} levels, were significantly increased in *nrt1.5* roots. Important genes including Na^+/H^+ *exchanger1*, Salt overly sensitive1, Pyrroline-5-carboxylate synthase1, Responsive to desiccation29A, Phytochelatin synthase1, and *NRT1.8* in stress response pathways are steadily up-regulated in *nrt1.5* mutant plants. Interestingly, altered accumulation of metabolites, including proline and malondialdehyde, was also observed in *nrt1.5* plants. These data suggest that NRT1.5 is involved in nitrate allocation to roots and the consequent tolerance to several stresses, in a mechanism probably shared with NRT1.8.

Nitrate is the most important nitrogen source for terrestrial plants. Nitrate concentrations in soil vary widely; thus, plants have evolved both a high-affinity transport system and a low-affinity transport system (Crawford, 1995), which correspond to NRT2 and NRT1 transporter families that function at low and high external nitrate concentrations, respectively (Daniel-Vedele et al., 1998; Stitt, 1999; Tsay et al., 2007). In Arabidopsis (*Arabidopsis thaliana*), there are 53 members in the NRT1 family, nine of which were identified as nitrate transporters to date. NRT1.1, also known as CHL1 (for chlorate resistance1), was the first one identified as a dual-affinity nitrate uptake transporter (Tsay et al., 1993; Wang et al., 1998; Liu et al., 1999), while the other eight show solely low-affinity transport activity (Tsay et al., 2007; Li et al., 2010; Wang and Tsay, 2011). Recent studies revealed that NRT1.1 is also a nitrate receptor that can sense a wide range of nitrate concentrations through phosphorylation regulation, thus regulating the nitrate primary response process (Liu and Tsay, 2003; Ho et al., 2009). *NRT1.1* and *NRT1.2* genes are expressed mainly in roots and regulate nitrate uptake from soil (Tsay et al., 1993; Huang et al., 1999). Another two responsible transporters are NRT2.1 and NRT2.2, which possibly represent the major uptake mechanism, as suggested by the dramatic decrease of nitrate uptake in their mutant plants (Filleur et al., 2001; Li et al., 2007).

Once taken up into roots, most nitrate undergoes long-distance transport to leaves and is assimilated in chloroplasts (Smirnoff and Stewart, 1985; Andrews, 1986). NRT1.5 and NRT1.8 have been identified as two essential transporters in nitrate long-distance transport (Lin et al., 2008; Li et al., 2010). The *NRT1.5* gene is expressed mainly in root pericycle cells and functions to load nitrate into xylem. Functional disruption of *NRT1.5* did not abort nitrate transport to aerial tissues, indicating that other xylem-loading transporter(s) may exist (Lin et al., 2008). *NRT1.8* is expressed predominantly in xylem parenchyma cells within the vasculature and functions to remove nitrate from xylem vessels. *NRT1.5* works together with *NRT1.8* to fine-tune nitrate long-distance transport from roots to shoots (Lin et al., 2008; Li et al., 2010).

Nitrate assimilation is an energy-intensive process, and many herbaceous plants tackle this problem by

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transporting nitrate to leaves, where energy and reductants derived from photosynthesis can be directly accessed by nitrate assimilation, thus rendering leaf nitrate assimilation more energy efficient than root assimilation (Canvin and Atkins, 1974; Smirnoff and Stewart, 1985; Andrews, 1986). However, it has long been observed that nitrate undergoes reallocation to roots when exposed to stresses, including low light and heavy metals (Smirnoff and Stewart, 1985; Hernandez et al., 1997). A recent study identified that the nitrate reallocation process is regulated by the induction of *NRT1.8* and contributes essentially to Cd^{2+} stress tolerance (Li et al., 2010). *NRT1.5* might also be involved in nitrate reallocation because it is down-regulated by Cd^{2+} stress (Zimmermann et al., 2004; Li et al., 2010); thus, nitrate retained in roots probably occurs based on the xylem nitrate-loading function of NRT1.5. Additionally, Web-based microarray analyses indicated that the coordinated opposite regulation patterns of *NRT1.8* and *NRT1.5* could be repeatedly observed under various biotic and abiotic stresses (Zimmermann et al., 2004; Li et al., 2010), leading to the hypothesis that nitrate reallocation in plants might be a common response to stresses (Gojon and Gaymard, 2010; Li et al., 2010), in which NRT1.5 might be another essential component. However, this hypothesis was established only with *NRT1.8* under Cd^{2+} stress so far; whether it is physiologically relevant to the *NRT1.5* gene or of general physiological significance remains to be determined.

In this study, several lines of experimental evidence are provided to demonstrate that NRT1.5 functions to mediate nitrate reallocation to roots, stress-responsive gene expression and metabolism, and consequently salt, drought, and Cd^{2+} tolerance, supporting the hypothesis that nitrate reallocation to roots might be a common response to stresses and is coordinately regulated by *NRT1.8* and *NRT1.5* genes.

RESULTS

Down-Regulation of *NRT1.5* by Various Stresses in Roots

To investigate if *NRT1.5* might play a role in the nitrate-regulated stress tolerance, reverse transcription (RT)-PCR was first performed to characterize *NRT1.5* expression using roots, as *NRT1.5* shows steadily high expression in roots but is undetectable in shoots under control conditions (Li et al., 2010). When exposed to NaCl, a significant decrease in *NRT1.5* expression level was observed 3 h after treatment in roots, and a further decrease occurred with prolonged treatment (Fig. 1A). Similar results were obtained for drought stress simulated by polyethylene glycol (PEG) application (Fig. 1B). Further quantitative RT-PCR analyses confirmed the down-regulation pattern of *NRT1.5* by various stresses in roots. Specifically, NaCl resulted in the most significant inhibition: a 12-h treatment reduced *NRT1.5* expression to one-tenth of the level under control conditions, while one-fifth of the control expression level was observed under 24-h drought stresses (Fig. 1D). Taken together, these data suggest that *NRT1.5* expression in Arabidopsis roots is down-regulated by various stresses, including NaCl, drought, and Cd^{2+}. Note that in shoots, the *NRT1.5* expression level is very low and not affected by stress treatments (Fig. 1D).

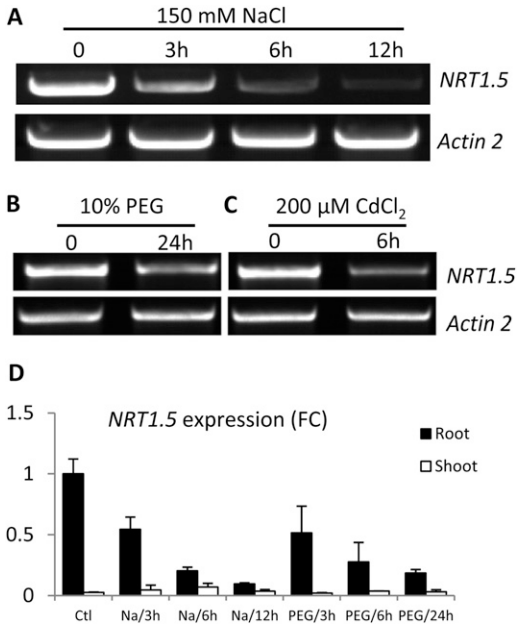

Figure 1. A to C, Down-regulation of *NRT1.5* by various stresses. Plants were grown hydroponically for 4 weeks and then exposed to 150 mM NaCl (A), 10% PEG (B), or 200 μM $CdCl_2$ (C) treatment for the indicated times. *NRT1.5* mRNA levels in roots were determined by RT-PCR. D, Quantitative RT-PCR determination of *NRT1.5* expression in both shoots and roots exposed to 200 mM NaCl or 10% PEG for the indicated times. The *y* axis shows fold change (FC) compared with the control (Ctl). Values are means ± SD (n = 3). *Actin2* was used as a loading control.

Enhanced Tolerance to Various Stresses in *nrt1.5* Mutants

Our previous work indicated that *NRT1.5* might contribute to Cd^{2+} tolerance and possibly other stress tolerance (Li et al., 2010). As expected, significantly enhanced Na^+ as well as Cd^{2+} tolerance was observed in the functional disruption mutants *nrt1.5-3* and *nrt1.5-4* compared with the wild-type ecotype Columbia (Col-0; Fig. 2, A and B), while no significant difference was seen between them when grown under control conditions (Fig. 2, A and B). Furthermore, less reduction of germination rate was observed in *nrt1.5* mutant seeds compared with the wild type, especially when under 200 mM NaCl treatment, where *nrt1.5-3* and *nrt1.5-4* seeds showed nearly 40% germination while Col-0 had less than 10% (Fig. 1C). Further analyses showed a significant increase in drought tolerance in *nrt1.5* mutant plants compared with Col-0 (Fig. 2D, middle and bottom panels), while similar growth was observed when under control conditions (Fig. 2D, top panel). Correspondingly, decreased water

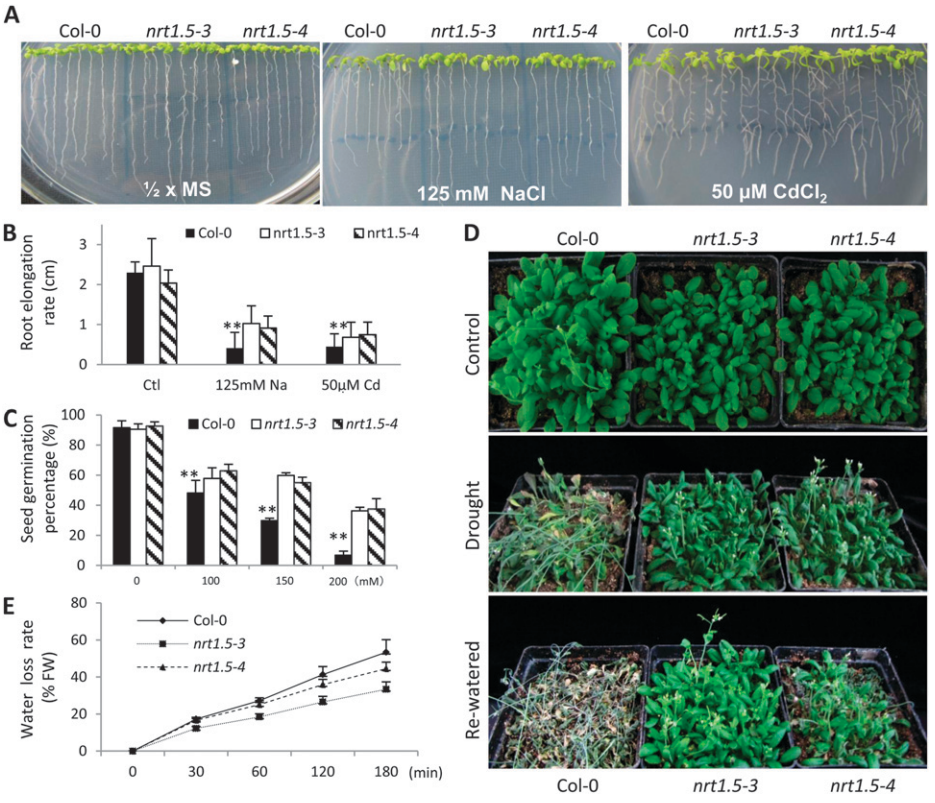

Figure 2. Enhanced tolerance to various stresses in *nrt1.5* mutants. A, Five-day-old seedlings were transferred to one-half-strength Murashige and Skoog medium (½ × MS) or one-half-strength Murashige and Skoog medium supplemented with 125 mM NaCl or 50 μM $CdCl_2$ and allowed another 6 d to grow. B, Root elongation between days 2 and 7 after transfer to the treatments in A. Values are means ± SD from three replicates, and each contained eight plants. ** $P < 0.01$. C, Seed germination rate on one-half-strength Murashige and Skoog medium supplemented with the indicated levels of NaCl. Values are means ± SD ($n = 200$ seeds). ** $P < 0.01$. D, Representative photographs for drought tolerance analysis of wild-type Col-0 and *nrt1.5* mutant plants. Control (top panel), drought-stressed (middle panel), and rewatered (bottom panel) plants were treated as described in "Materials and Methods" before imaging. E, Water loss rate of leaves detached from 16-d-old plants. Values are means ± SD ($n = 15$). FW, Fresh weight.

loss rates were detected in *nrt1.5* plants compared with the wild-type control (Fig. 2E). Furthermore, when replacing nitrate with ammonium, the enhanced stress tolerance in *nrt1.5* mutants was abandoned (Supplemental Fig. S1). These data indicate that the down-regulation of *NRT1.5* mediates tolerance to various stresses in a nitrate-dependent manner.

Increased Nitrate Allocation to Roots of *nrt1.5* Mutants

It has been reported that *NRT1.8* induction in Arabidopsis increases nitrate allocation to roots, thus enhancing Cd^{2+} tolerance (Li et al., 2010). Given that down-regulation of the nitrate transporter gene *NRT1.5* in roots also enhanced tolerance to various stresses (Fig. 2), we tested whether a similar nitrate reallocation could be observed in *nrt1.5* mutant plants.

Under control conditions, nitrate levels in shoots of *nrt1.5-3* and *nrt1.5-4* were significantly lower than in Col-0, while more were detected in roots (Fig. 3A), which translates to a root-shoot nitrate ratio of 0.28 in the wild type but 0.51 in *nrt1.5* mutant plants, consistent with previous results (Lin et al., 2008). Salt stress significantly decreased the overall nitrate contents in both wild-type and mutant plants; however, proportionally more nitrate was still detected in roots of *nrt1.5* than those of Col-0 (Fig. 3A). Similar results were obtained in plants grown under drought and Cd^{2+} stress conditions (Fig. 3, B and C). These data demonstrated that functional disruption of *NRT1.5* constitutively impaired xylem nitrate loading, and thus more nitrate was retained in roots. Salt and drought stresses did not further increase nitrate reallocation to roots in *nrt1.5* mutants, possibly because the disruption of *NRT1.5* renders nitrate reallocation no longer a response to stresses but a constitutive alteration. In contrast, Cd^{2+} treatment increased the root-shoot nitrate ratio from 0.46 to 0.48 under control conditions to 0.59 to 0.66 under Cd^{2+} stress, indicating that more complicated mechanisms may be involved in regulating nitrate reallocation under Cd^{2+} stress.

Given that further nitrate allocation could not be easily observed in *nrt1.5* mutants when exposed to stresses, we then tested the stress sensitivity in *nrt1.8-1*, which could enhance nitrate allocation to shoots, thus resembling *NRT1.5* overexpression. As expected, significantly increased drought sensitivity was observed in *nrt1.8-1* compared with the wild-type ecotype Wassilewskija (Ws), and crossing *nrt1.8-1* to *nrt1.5* neutralized the drought tolerance observed in *nrt1.5* plants to a level comparable to the wild-type control (Supplemental Fig. S2A). Similar results were observed when *nrt1.8-1* and the double mutants were exposed to salt stress (Supplemental Fig. S2, B–E). These data suggest that nitrate allocation regulated either by NRT1.5 or NRT1.8 is essential to stress tolerance. Note that a different growth medium was used for *nrt1.5* and *nrt1.8* mutants in the salt tolerance assay, as similar growth between *nrt1.5* mutants and their wild type under control conditions could be obtained only when

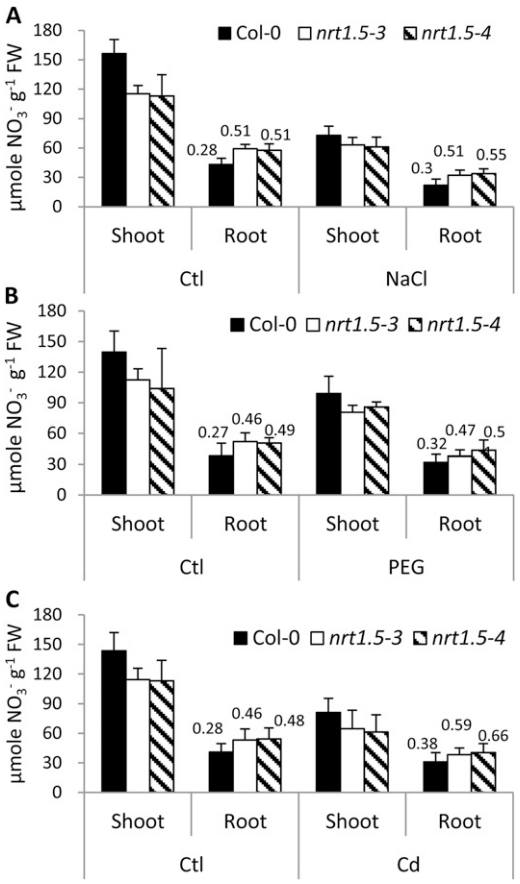

Figure 3. Increased nitrate allocation to roots of *nrt1.5* mutants. Four-week-old hydroponically grown plants were treated by 50 mM NaCl for 3 d (A), 10% PEG for 24 h (B), 20 μM $CdCl_2$ for 3 d (C), or under control conditions (Ctl). Then, shoot and root tissues were harvested and subjected to determination of nitrate concentration by HPLC. The numbers above each bar represent root-shoot nitrate ratio. Values are means ± SD from three replicates, and each pooled more than nine plants. FW, Fresh weight.

the nitrate level paralleled those of other nutrients, and stress tolerance in *nrt1.5* mutants depended less on nitrate concentration (Figs. 2, A and B; Supplemental Fig. S5).

Altered Na^+ or Cd^{2+} Distribution in *nrt1.5* Mutants

A previous study showed that in the *nrt1.8* mutant, proportionally more nitrate accumulated in shoots, where correspondingly more Cd^{2+} was detected (Li et al., 2010). Consistent with this observation, a similar positive correlation between nitrate and metal accumulation was also observed in *nrt1.5-3* and *nrt1.5-4* plants. Na^+ accumulation in *nrt1.5* shoots was significantly decreased compared with Col-0 while increased Na^+ contents were detected in roots; the root-shoot Na^+ content ratios were 0.59 and 0.58 in *nrt1.5-3* and *nrt1.5-4*, respectively, in contrast to approximately 0.36 in Col-0 (Fig. 4A). Furthermore, the Na^+ concentration in xylem sap from *nrt1.5* mutant plants was significantly decreased compared with that from Col-0 (Fig. 4C). A similar Cd^{2+} distribution between roots and shoots was also observed in *nrt1.5* mutant plants (Fig. 4, B and D). Given that functional disruption of *NRT1.5* retains more nitrate in roots (Fig. 3) and decreases nitrate concentration in xylem sap (Li et al., 2010), these data suggest that whether regulated by *NRT1.8* or *NRT1.5*, nitrate reallocation leads to increased metal accumulation in tissues where nitrate concentration increases. Note that although Na^+ and Cd^{2+} were provided as chloride salts, a positive correlation between Cl^- and NO_3^- could not be observed in *nrt1.5* mutants (Supplemental Fig. S4), in contrast to that between Na^+/Cd^{2+} and NO_3^- (Fig. 4), suggesting that the stress tolerance in *nrt1.5* mutants might not be ascribed to Cl^- allocation.

Functional Disruption of *NRT1.5* Alters the Expression of Stress-Related Genes

To investigate the underlying mechanisms of enhanced stress tolerance in *nrt1.5* mutant plants (Fig. 2), the expression levels of marker genes in stress response pathways were determined. Under control conditions, *NHX1*, High-affinity K^+ transporter1 (*HKT1*), Salt overly sensitive1 (*SOS1*), and *SOS2* showed steadily higher expression in *nrt1.5* than in Col-0 (Fig. 5A). Further increased expression of these genes, especially of *NHX1*, *HKT1*, and *SOS1*, which are responsible for vacuolar Na^+ sequestration, long-distance Na^+ transport, and Na^+ efflux, respectively (Shi et al., 2000; Yokoi et al., 2002; Berthomieu et al., 2003), was observed in *nrt1.5* plants than in the wild type when treated by salt stress (Fig. 5A).

Among the selected drought tolerance-related genes, *P5CS1* and *RD29A* were most significantly affected by *NRT1.5* (Fig. 5B). In shoots of *nrt1.5*, steadily higher expression of *P5CS1* was observed than in Col-0 under control conditions, and further increases were detected in both shoots and roots of *nrt1.5* when exposed to drought stress (Fig. 5B). Similar results were obtained for *RD29A*, in which drought stress further increased its relatively higher expression level in *nrt1.5* than in Col-0 under control conditions (Fig. 5B). In terms of the Cd^{2+} response pathway, significantly higher expression of *AtPCS1* was observed in *nrt1.5* roots under Cd^{2+} stress (Fig. 5C). Further quantitative RT-PCR analyses confirmed these results (Fig. 5, D and E). Taken together, these data suggest that nitrate reallocation affects the expression levels of several genes in stress response pathways, and these genes, including *SOS1*, *NHX1*, *P5CS1*, *RD29A*, and *AtPCS1*, might be used as biomarkers to further investigate the interaction between nitrate reallocation and stress tolerance.

Interestingly, even under control conditions, significantly increased expression of *NRT1.8* was observed

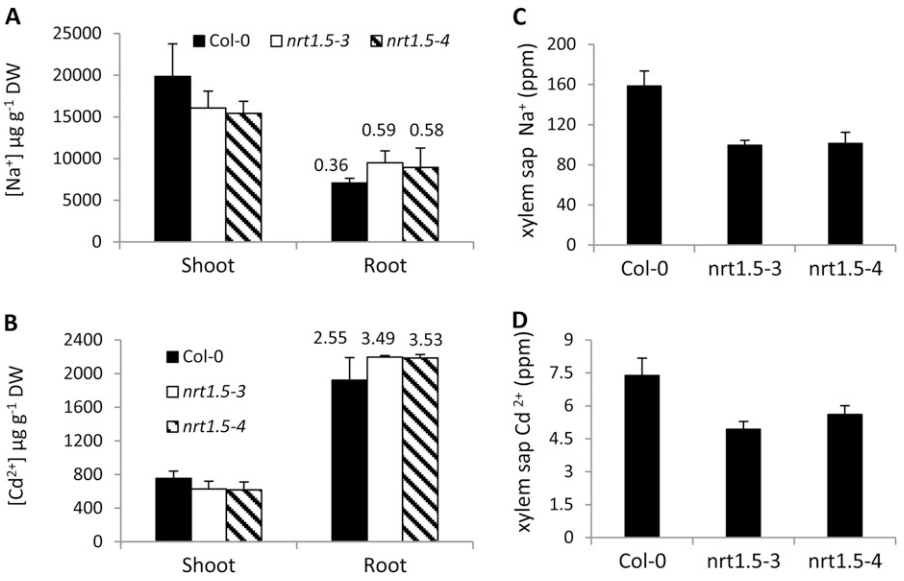

Figure 4. Altered Na^+ or Cd^{2+} distribution in *nrt1.5* mutants. A and B, Increased Na^+ or Cd^{2+} accumulation in *nrt1.5* roots. Four-week-old plants were exposed to 50 mM NaCl (A) or 20 μM $CdCl_2$ (B) for 3 d before shoots and roots were sampled. The numbers above each bar represent root-shoot ratio of Na^+ or Cd^{2+}. C and D, Decreased Na^+ or Cd^{2+} concentration in xylem sap. Four-week-old plants were treated with 10 mM NaCl for 1 d (C) or 5 μM $CdCl_2$ for 3 d (D) before xylem sap was collected. Values are means ± SD from three (A and B) or nine (C and D) replicates. DW, Dry weight.

in *nrt1.5* mutants, and dramatic increases occurred in roots of both mutants and in shoots of *nrt1.5-3* (Supplemental Fig. S3A). Stress treatments did not show any further effect on *NRT1.8* expression in *nrt1.5* mutants, except in *nrt1.5-3* shoots (Supplemental Fig. S3A). In contrast to *NRT1.5* expression in the *nrt1.8-2* mutant, the *nrt1.8-1* mutant with a replaced genetic background of Col-0 had similar expression to that in its wild-type control (Supplemental Fig. S3B) and appeared not to respond to stresses (data not shown). These data suggest that the down-regulation of *NRT1.5* occurs prior to the induction of *NRT1.8* in roots exposed to stresses.

Altered Accumulation of Stress-Related Metabolites in *nrt1.5*

Pro functions as an important osmolyte and contributes essentially to stress tolerance in higher plants. In this study, the expression of *P5CS1*, the essential regulatory gene in the Pro biosynthesis pathway (Kishor et al., 2005; Székely et al., 2008), was significantly

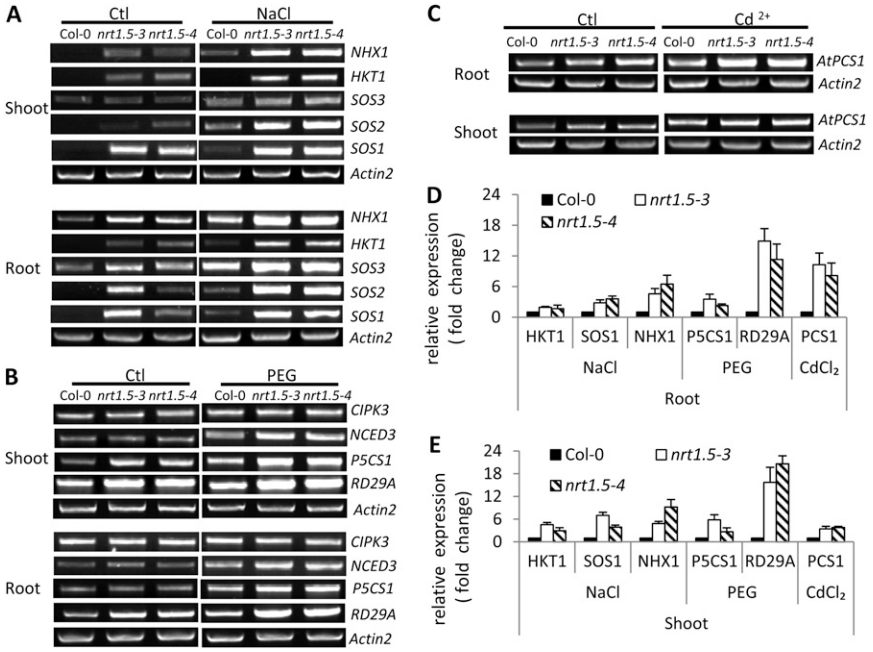

Figure 5. A to C, Functional disruption of *NRT1.5* alters the expression levels of stress-related genes. Four-week-old hydroponically grown plants were treated with 200 mM NaCl for 6 h (A),10% PEG for 24 h (B), 200 μM $CdCl_2$ for 6 h (C), or under control conditions (Ctl). D and E, RT-PCR was performed for selected stress-related marker genes. Quantitative RT-PCR determination of representative gene expression in roots (D) and shoots (E). Values are means ± SD (n = 3). *Actin2* was used as a loading control.

altered by functional disruption of *NRT1.5* (Fig. 5B), which leads to the notion that the Pro level might also be changed in *nrt1.5* mutants. Further analyses showed that under control conditions, Pro levels were slightly higher in *nrt1.5* than in wild-type Col-0, while Na^+ stress significantly increased Pro contents in *nrt1.5* compared with Col-0, and the most significant increase was observed with 150 mM NaCl treatment for 1 d (Fig. 6A). Similar results were obtained in drought-treated plants (Fig. 6B). These data suggest that nitrate allocation to roots enhances Pro synthesis and accumulation.

Malondialdehyde is one of the major lipid oxidation products (Esterbauer et al., 1991; Weber et al., 2004), which indicates how severely plants are stressed (Zheng et al., 2008). Corresponding to the increased Pro level in *nrt1.5* plants (Fig. 6, A and B), relatively decreased malondialdehyde levels were detected in *nrt1.5* plants compared with Col-0 under salt and drought stresses (Fig. 6, C and D), indicating that nitrate allocation to roots decreases oxidative stress in plants.

DISCUSSION

NRT1.5 Is Another Essential Component in Regulating Nitrate Reallocation to Roots and the Consequent Stress Tolerance

Our previous study showed that nitrate redistribution contributes essentially to Cd^{2+} tolerance in Arabidopsis, and this process is actively regulated by the xylem nitrate-unloading transporter NRT1.8. Interestingly, NRT1.5, the xylem nitrate-loading transporter, was significantly down-regulated by Cd^{2+}, leading to the hypothesis that NRT1.5 may also contribute to the nitrate reallocation to roots, hence enhancing plant stress tolerance (Li et al., 2010). Here, we present data to show that in Col-0, the root-shoot nitrate ratios were 0.28 to 0.38, while in *nrt1.5*, the value varied from 0.46 to 0.66 (Fig. 3), indicating that proportionally more nitrate accumulated in *nrt1.5* roots compared with wild-type Col-0. Moreover, *nrt1.5* showed enhanced tolerance to Cd^{2+}, salt, and drought stresses (Fig. 2). These data together suggested that down-regulation of *NRT1.5* by various stresses helps to retain nitrate in roots and contributes essentially to stress tolerance in a similar mechanism to that proposed for *NRT1.8* (Li et al., 2010).

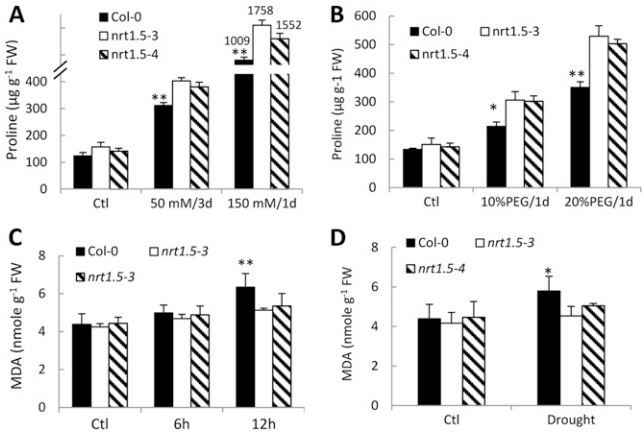

Figure 6. Stress-related metabolite accumulation in *nrt1.5* mutants. Four-week-old plants were exposed to the indicated NaCl treatments (A), PEG treatments (B), or 150 mM NaCl (C) for the indicated times, 10% PEG for 1 d (D), or under control conditions (Ctl). Shoots were sampled for the determination of Pro or malondialdehyde (MDA) levels. Values are means ± SD ($n = 6$). ** $P < 0.01$. FW, Fresh weight.

The Role of Nitrate Reallocation in Regulating Stress Tolerance

It has been believed that nitrate long-distance transport to shoots allows plants more energy efficiency during evolutionary competition, although nitrate reallocation to roots is frequently observed under adverse conditions (Canvin and Atkins, 1974; Smirnoff and Stewart, 1985; Andrews, 1986; Ericsson, 1995; Hernandez et al., 1997). In our studies, either the up-regulation of *NRT1.8* or the down-regulation of *NRT1.5* enhances plant tolerance to Cd^{2+} stress, establishing that no matter the molecular basis, nitrate reallocation to roots represents an essential mechanism regulating Cd^{2+} tolerance (Li et al., 2010; Fig. 2) rather than a passive consequence of inhibited transpiration rate (Hernandez et al., 1997).

Furthermore, the opposite expression pattern of *NRT1.8* and *NRT1.5* is regulated not only by Cd^{2+} but by a wide range of stresses, including abiotic and biotic stresses (Zimmermann et al., 2004; Li et al., 2010), implying that nitrate reallocation may be a universal mechanism in regulating various stress tolerances than a specific response to Cd^{2+} (Gojon and Gaymard, 2010; Li et al., 2010). An extended assay on *nrt1.5* mutant plants under salt and drought stresses provided experimental evidence for this hypothesis, because increased nitrate accumulation in *nrt1.5* roots (Fig. 3) significantly enhanced drought and salt tolerance in *nrt1.5* (Fig. 2). It is worth noting that, in contrast to the wild type, *nrt1.5* mutants show a complete absence of *NRT1.5*; thus, nitrate allocation is no longer a response to stresses but a constant alteration (Fig. 3), which resembles stress pretreatment and would possibly lead to a wide range of physiological consequences, as was observed in seed germination and water loss rate in *nrt1.5* mutants (Fig. 2). Further support for this hypothesis came from the result that, in addition to other important genes, *NRT1.8* shows constitutive high expression in *nrt1.5* mutants (Fig. 5; Supplemental Fig. S3A).

Possible Mechanisms of Stress Tolerance Regulated by Nitrate Reallocation, and Prospects of Future Research

Nitrate reallocation to roots was proposed to serve as a signal to regulate Cd^{2+} tolerance in Arabidopsis (Li et al., 2010), mainly based on the following observations: (1) nitrate represents only a minor proportion of all the nitrogen subjected to reallocation to roots, so the nutritional effect is subtle and insignificant; (2)

extensive changes of gene expression in *NRT1.8* overexpression lines; and (3) the Cd^{2+} distribution between roots and shoots was altered and showed a positive correlation with nitrate levels, although NRT1.8 appears unable to transport either Cd^{2+} or its major chelators, phytochelatins and glutathione.

Consistently, nitrate reallocation in *nrt1.5* extensively altered the expression levels of many marker genes in drought, salt, and Cd^{2+} response pathways (Fig. 5), especially the levels of *HKT1* and *SOS1*, which regulate Na^+ distribution, and *P5CS1* and *AtPCS1*, which regulate osmolyte synthesis and Cd^{2+} distribution, respectively (Strizhov et al., 1997; Shi et al., 2000; Gong et al., 2003, 2004; Sunarpi et al., 2005; Pomponi et al., 2006; Székely et al., 2008). Indeed, Cd^{2+} and Na^+ distribution in roots and shoots was altered correspondingly (Fig. 4). Moreover, significant changes were also observed in Pro and malondialdehyde levels (Fig. 6), indicating that *nrt1.5* mutants adapt to stresses more efficiently than the wild type by altering osmolyte and reactive oxygen species metabolism, consistent with the results that several genes in the stress response pathway show steadily higher expression in *nrt1.5* than in Col-0 (Fig. 5). Given that these alterations derive from different environmental cues and are involved in gene expression (Fig. 5), solute transport (Fig. 4), and secondary metabolism (Fig. 6), the most simple interpretation would be that nitrate reallocation serves as a signal to bridge various stress cues and extensive physiological changes, consequently enhancing stress tolerance.

As to how exactly nitrate reallocation regulates stress tolerance, it remains largely to be investigated. In tobacco (*Nicotiana tabacum*), altered nitrate allocation to shoots had been suggested as a signal to coordinately reprogram nitrogen and carbon metabolism and significantly decreased sugar contents in roots (Scheible et al., 1997a, 1997b), indicating that sugar allocation and signaling might be part of the nitrate signaling pathway. Furthermore, in studies of nitrate reallocation under stress, an interesting observation is that *NRT1.8* and *NRT1.5* are oppositely regulated by various stresses (i.e. *NRT1.8* is up-regulated while *NRT1.5* is reduced; Zimmermann et al., 2004; Li et al., 2010), which eventually results in the similar consequence of retaining nitrate in roots. This specific regulation pattern raises the concern that whether *NRT1.8* and *NRT1.5* share a common regulation mechanism, the down-regulation of *NRT1.5* may be actively regulated, or just a passive consequence of stresses, or even a feedback response of sugar allocation. The fact that *NRT1.8* expression is constitutively up-regulated in *nrt1.5* mutants suggests that the down-regulation of *NRT1.5* might be an active process that functions genetically upstream of *NRT1.8* (Supplemental Figs. S2 and S3); thus, *NRT1.5* and *NRT1.8* might share a similar downstream mechanism to regulate stress tolerance. This hypothesis is consistent with the altered gene expression pattern and solute transport observed in both *nrt1.5* mutants and *NRT1.8* transgenic lines (Figs. 4 and 5; Li et al., 2010). Further study on how *NRT1.5* is down-regulated by stresses might shed light on and essentially promote the understanding of the stress-initiated nitrate signaling pathway.

In summary, functional disruption of the *NRT1.5* gene enhanced nitrate accumulation in roots and tolerance to Cd^{2+}, Na^+ and drought stresses, consistent with the model that the opposite regulation of *NRT1.8* and *NRT1.5* serves as the essential molecular basis to regulate nitrate reallocation to roots. Our research further supports the hypothesis that nitrate allocation to roots is a common mechanism in regulating a wide range of stresses.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) Col-0 plants were used as the wild-type control for *nrt1.5* mutants, while Ws was used as the control for *nrt1.8-1* mutants. Crossing of *nrt1.8* into *nrt1.5* was performed to generate the homozygous double mutants *dm1* (*nrt1.5-3/nrt1.8-1*) and *dm2* (*nrt1.5-4/nrt1.8-1*), and the hybrids (C×W) from Col-0 and Ws plants were used as the wild-type control. The mutant *nrt1.8-2* in the Col-0 genetic background was generated by crossing *nrt1.8-1* (in the Ws background) into Col-0 five times.

Growth Conditions and Stress Sensitivity Analyses

Arabidopsis plants were grown in one-quarter-strength sterile hydroponic solution at 22°C with a 16-h-light/8-h-dark cycle as described (Arteca and Arteca, 2000; Gong et al., 2003). At 3 to 4 weeks of age, plants were exposed to treatments as indicated in the legends of Figures 1 and 3 to 6 and Supplemental Figures S3 and S4. For sensitivity analyses of plants on plates, seedlings were grown for 5 to 6 d on one-half-strength Murashige and Skoog basal medium (Sigma-Aldrich) with 1 g L^{-1} MES, 0.8% (w/v) Suc, and 1.5% Bacto agar or on one-quarter-strength minimal medium (Arteca and Arteca, 2000; Gong et al., 2003) when indicated. They were then transferred to plates with basal medium or medium supplemented with NaCl or $CdCl_2$ at the indicated concentrations and allowed to grow vertically for another 6 to 7 d, at which point root elongation was determined. For the seed germination assay, Arabidopsis seeds were plated and allowed 4 d of incubation before the germination rate (percentage of germinated seeds) was determined. For the drought tolerance assay, wild-type and mutant plants grown in soil were irrigated for 12 d (control) and then drought stressed by terminating irrigation for 12 d for Col-0 and *nrt1.5*, 8 d for *nrt1.8* and Ws, or 10 d for the double mutants *dm1* and *dm2* and the control plant C×W. Photographs of rewatered plants were taken 3 to 5 d after rewatering. For the water loss assay, wild-type (Col-0) and *nrt1.5* mutant plants were irrigated normally for 16 d and then fully expanded rosette leaves were sampled; plant fresh weight was determined at the indicated time intervals to calculate the percentage of fresh weight decrease.

Nitrate-Dependent Assay

In the nitrogen source-dependent assay, KNO_3 and $Ca(NO_3)_2$ in one-quarter-strength minimal medium were replaced with 1.25 mM KCl and 0.5 mM $CaCl_2$, and then 5 mM filter-sterilized ammonium succinate was added as the sole nitrogen source to make ammonium plates. Seedlings were germinated on ammonium plates with 0.8% (w/v) Suc and 1.5% Bacto agar and incubated vertically for 7 d; then they were transferred to the minimal ammonium plates (without Suc) or plates supplemented with either 50 mM NaCl or 50 μM $CdCl_2$. Alternatively, for the nitrate concentration assay, wild-type and *nrt1.5* plants were grown in one-quarter-strength minimal medium with 0.8% (w/v) Suc and 1.5% Bacto agar for 5 d, and then the seedlings were transferred to minimal medium with nitrate at the indicated concentration with or without 50 mM NaCl or 50 μM $CdCl_2$. Photographs were taken 6 d later, and root elongation between days 2 and 7 after transfer was determined.

RT-PCR and Quantitative RT-PCR

Four-week-old hydroponically grown plants were exposed to NaCl, PEG (PEG6000), or $CdCl_2$ (Fig. 1, Fig. 5, and Supplemental Fig. S3). Total RNA was extracted from roots and shoots using TRIzol reagent (Invitrogen) following the manufacturer's instructions. First-strand complementary DNA was synthesized from DNaseI-digested total RNA using Moloney murine leukemia virus reverse transcriptase (Promega), and PCR was performed on a Perkin-Elmer GeneAmp 9700 with the indicated cycles using Ex-Taq DNA polymerase (TaKaRa); PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Quantitative RT-PCR was performed on a Corbett Research Rotor-Gene 3000 thermal cycler using SYBR Premix Ex-Taq (TaKaRa) according to the manufacturers' protocols.

Determination of Nitrate, Chloride, Na^+, Cd^{2+}, and Metabolite Levels

Four-week-old hydroponically grown plants were treated as indicated in the legends of Figures 3, 4, and 6 and Supplemental Figure S4. Shoots and roots were sampled as described (Li et al., 2010). Nitrate was extracted in boiling water and determined by HPLC (Agilent 1200 series) using a PARTISIL 10 strong anion-exchange column (Whatman) as described (Chiu et al., 2004). Chloride was extracted by deionized water and filtered through a C18 pre-column filter (Sigma-Aldrich). Chloride content was determined by ion chromatography (Agilent IC5000 series) as described by Kong et al. (2011) with minor modifications. Metal accumulation was determined using inductively coupled plasma-mass spectrometry (Elan DRC-e; Perkin-Elmer) as described (Gong et al., 2003) with minor modifications. To collect enough xylem sap, plants were exposed to mild treatments (Fig. 4). Xylem sap was collected for 6 h as described (Sunarpi et al., 2005). Alternatively, plants were treated with salt and drought stresses before sampling for the determination of Pro concentration by ninhydrin colorimetry (Bates et al., 1973; Sharma and Dubey, 2005) or the determination of malondialdehyde using the thiobarbituric acid method with minor modifications (Kramer et al., 1991; Zheng et al., 2008).

Statistical Analyses

Two-tailed Student's *t* tests were performed to compare fresh weights and nitrate, Na^+, Cd^{2+}, and metabolite contents between mutant and wild-type plants. Differences were deemed significant at $P < 0.05$ and extremely significant at $P < 0.01$.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g32450 (NRT1.5), At4g21680 (NRT1.8), At1g12110 (CHL1), At1g69850 (NRT1.2), At1g08090 (NRT2.1), At1g08110 (NRT2.2), AT5G44070 (PCS1), AT5G52310 (RD29A), AT2G39800 (P5CS1), AT2G26980 (CIPK3), AT3G14440 (NCED3), AT5G27150 (NHX1), AT4G10310 (HKT1), AT2G01980 (SOS1), AT5G35410 (SOS2), AT5G24270 (SOS3), and AT3g18780 (Actin2).

Supplemental Data

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

Supplemental Figure S1. Enhanced stress tolerance in *nrt1.5* mutants is nitrate dependent.

Supplemental Figure S2. *nrt1.8* is sensitive to drought and salt stresses and neutralizes phenotypes observed in *nrt1.5* mutants.

Supplemental Figure S3. Increased *NRT1.8* expression in *nrt1.5* mutants.

Supplemental Figure S4. Chloride allocation in *nrt1.5* mutants.

Supplemental Figure S5. Nitrate-dependent growth of *nrt1.5* mutants.

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