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LeNRT2.3 functions in nitrate acquisition and long-distance transport in tomato

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ABSTRACT

Nitrogen plays an important role in plant growth and development. Nitrate transporters have been extensively studied in *Arabidopsis*, but in tomato they have not been functionally characterized. In this study, we report the functions of *LeNRT2.3* in nitrate transport in tomato. Our results show that *LeNRT2.3* is induced by nitrate, and mainly localizes to the plasma membranes of rhizodermal and pericycle cells in roots. Further analysis in *Xenopus* oocytes showed that LeNRT2.3 mediates low-affinity nitrate transport. *35S:LeNRT2.3* increased nitrate uptake in root and transport from root to shoot. More interestingly, *35S:LeNRT2.3* showed high biomass and fruit weight. Taken together, these results suggest that *LeNRT2.3* plays a double role in nitrate uptake and long-distance transport in tomato.
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1. Introduction

Nitrate (NO_3^-) concentration varies drastically in the soil, hence plants adopt two types of transport systems to take up NO_3^-, including low-affinity transport systems (LATS) and high-affinity transport systems (HATS) [1–4]. When external nitrate concentration is high (>1 mM), LATS contributes substantially to nitrate uptake, while HATS is activated at low NO_3^- concentration [1,4–6].

Two families of nitrate transporters, NPF/NRT1 and NRT2, have been identified in *Arabidopsis*, which are responsible for LATS and HATS, respectively [6]. Among the 53 members of the NPF/NRT1 family, AtNPF6.3/NRT1.1 was identified as a dual-affinity transporter, and the dual-affinity uptake is realized by phosphorylation and dephosphorylation [7–9]. Other characterized NPF/NRT1 transporters showed a broad range of substrate selectivity [10].

The *NRT2* family consists of 7 members in *Arabidopsis*. AtNRT2.1, AtNRT2.2, AtNRT2.4 and AtNRT2.5 are involved in high-affinity nitrate uptake [11–13]. AtNRT2.4 plays a double role in nitrate uptake in roots and phloem NO_3^- transport in shoots [12]. AtNRT2.5 takes part in nitrate uptake in roots and loading into the phloem during nitrate remobilization [13]. In rice, five NRT2 members have been identified [14–16]. OsNRT2.1, OsNRT2.2, and OsNRT2.3a affect nitrate transport interact with OsNAR2.1 [16]. In barley, four members of NRT2 family have been isolated [17,18]. HvNRT2.1 transports nitrate with HvNAR2.3 [19]. In *Chlamydomonas reinhardtii*, CnNRT2.1 and CnNRT2.2 act in high affinity nitrate transport [20].

Once transported into roots, nitrate is either stored in vacuoles, or assimilated to organic nitrogen and partitioned to plastids [21]. Alternatively, nitrate is loaded into xylem vessels and transported to the aerial parts [22]. AtNPF7.3/NRT1.5, AtNPF7.2/NRT1.8 and AtNPF2.9/NRT1.9 participate in the step of nitrate long-distance transport. NRT1.5 is expressed in pericycle cells, and loads nitrate into xylem [23]. AtNPF7.2/NRT1.8 is expressed in xylem parenchyma cells, and unloads nitrate from xylem [24]. AtNPF2.9/NRT1.9 is expressed in phloem companion cells, removes nitrate from the xylem sap and acts in shoot-to-root transport of nitrate [25].

Tomato is one of the most economically important vegetable crops in the world. As the major nitrogen resource, nitrate plays

Abbreviations: LATS, low-affinity transport systems; HATS, high-affinity transport systems; NRT, nitrate transporter

Authors contributions: Yan-lei Fu and Ji-ming Gong designed the research. Yan-lei Fu, Hong-ying Yi and Juan Bao performed the research and analyzed the data. And Yan-lei Fu wrote the article.

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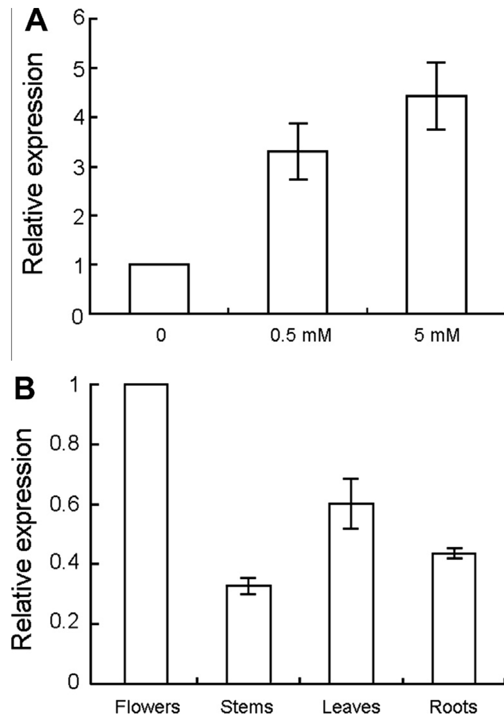

Fig. 1. *LeNRT2.3* is nitrate responsive in tomato. (A) 24-day-old plants grown hydroponically were treated with 0, 0.5 and 5 mM nitrate for 4 d. The expression levels of *LeNRT2.3* were determined by quantitative realtime-RT-PCR. (B) Relative *LeNRT2.3* mRNA expression levels in different tissues. *GAPDH* was used as an internal control. Values are mean ± S.E., $n = 3$.

an important role in plants growth and development. However, the molecular mechanisms of nitrate uptake in roots and long-distance transport are poorly understood. So far, only five genes, *LeNRT1.1*, *LeNRT1.2*, *LeNRT2.1*, *LeNRT2.2* and *LeNRT2.3* are identified. The five genes are all expressed in roots and induced by nitrate [26–28], but none of the genes are functionally studied.

In this study, we showed that LeNRT2.3 is a plasma membrane localized nitrate transporter implicated in two processes, uptake of nitrate in roots and transport of nitrate from root to shoot. This dual role of LeNRT2.3 possibly allows tomato to utilize nitrate more efficiently.

2. Materials and methods

2.1. Plant materials and growth conditions

Tomato (*Solanum lycopersicum*) ecotype Micro-Tom was used as the wild-type controls. The seeds were germinated and grown on half-strength MS plates for 7 d before being transferred to hydroponics under long-day conditions (16-h light/8-h dark) at 22 °C. Plants were grown in half-strength MS hydroponics to 4 weeks of age, and exposed to nitrate treatments as indicated.

2.2. DNA constructs and plant transformation

The *LeNRT2.3* cDNA was amplified by RT-PCR. The two restriction sites for *Bam*HI and *Spe*I were introduced using *LeNRT2.3*-1 primers (forward, 5′-ggatccatgggtgatattgaaggat-3′; reverse, 5′-acta gtcagacgcgatttggtgtta-3′). The resulting fragments were confirmed by sequencing and then subcloned into the binary vector pBI121 (predigested with *Bam*HI and *Spe*I). Tomato cotyledon explants were transformed with agrosuspension essentially as described [29]. Transgenic lines were used to further screen homozygotes and strong alleles with a segregation rate of 3:1 grown on kanamycin plates.

2.3. Quantitative RT-PCR

Total RNA was isolated from plants grown under the indicated conditions using TRIzol reagent. First-strand cDNA synthesis, quantitative RT-PCR were performed as previously described [24]. The primers used were as follows: *GAPDH* (forward, 5′-ctgctct ctcagtagccaacac-3′; reverse, 5′-cttcctccaatagcagaggttt-3′) and *LeNRT2.3*–2 (forward, 5′-tgtacacttccagtaatgttagtt-3′; reverse, 5′-gg tacccagacgcgatttggtgtta-3′).

2.4. In situ hybridization

One-week-old tomato seedlings were transferred to nitrogen-depleted medium for 3 d from half-strength MS medium. Then they were subjected to nitrate induction as indicated for 4 d. Tissue sectioning, digoxigenin labeling of RNA probe, and in situ hybridization were performed as described [24,30]. A gene-specific fragment containing the 1596-bp (1–1596) coding region of *LeNRT2.3* was amplified by PCR and cloned into pGEM T Easy vector (Promega). Sense and antisense probes were

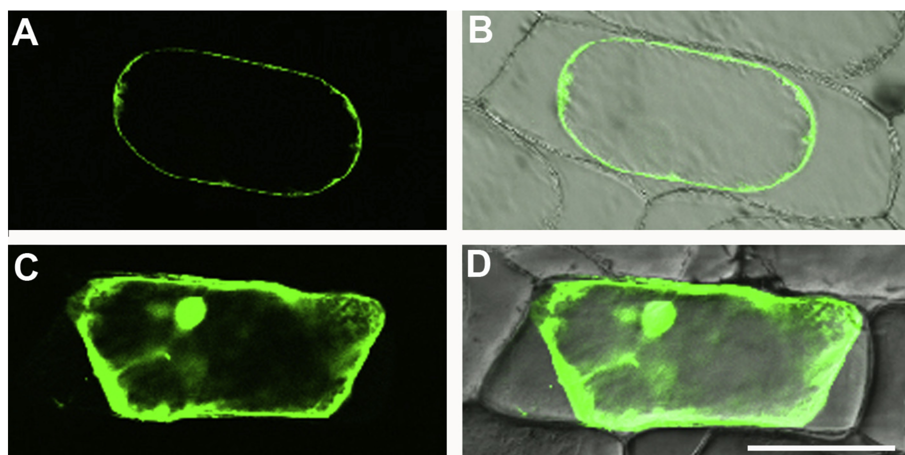

Fig. 2. *LeNRT2.3* is subcellular located to the plasma membrane. (A) Fluorescence image of epidermal cell expressing the EYFP:LeNRT2.3 fusion protein. (B) Merged control EYFP fluorescence and bright-field image. (C) Fluorescence image of epidermal cell expressing EYFP as a control. (D) Merged EYFP fluorescence and bright-field image. Bars = 100 μm in A–D.

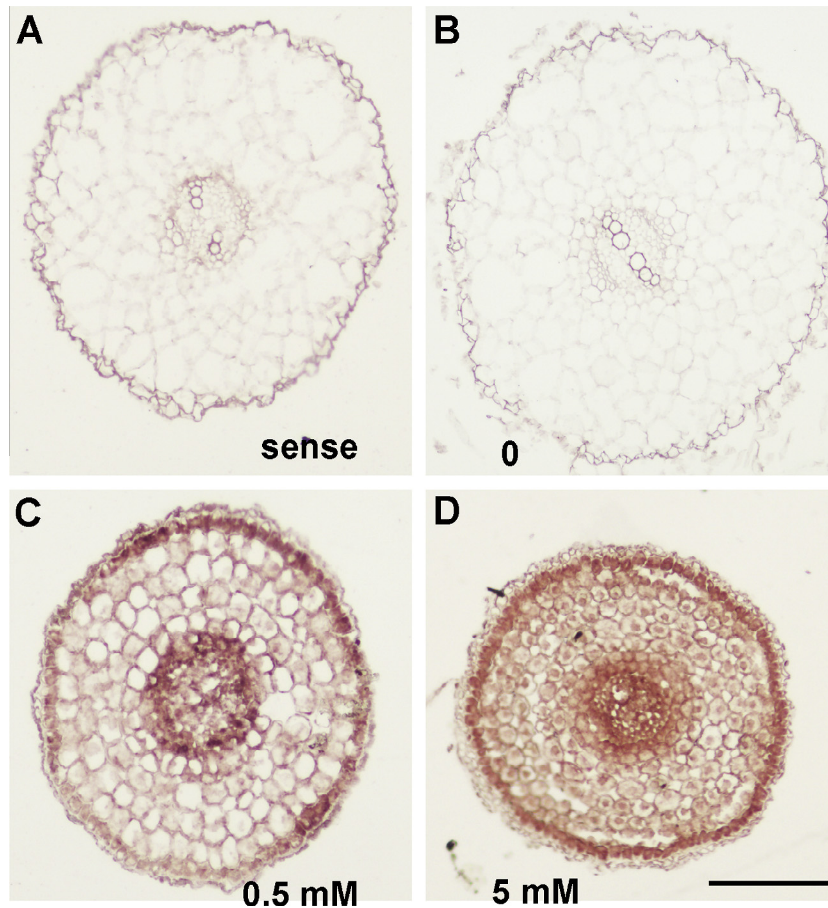

Fig. 3. *LeNRT2.3* is expressed in rhizodermal and pericycle cells. (A) In situ hybridization of the sense *LeNRT2.3* probe to a section of tomato root tissue. (B)–(D) In situ hybridization of the antisense *LeNRT2.3* probe to a section of tomato root tissue treated with 0 (B), 0.5 (C) and 5 mM nitrate (D). Bars = 0.2 mm in A–D.

in vitro synthesized using T7 and SP6 primers according to the manufacturer's instructions.

2.5. EYFP fusion and subcellular localization

The cDNA of *LeNRT2.3* was amplified by PCR to introduce *Xho*I and *Eco*RI sites. This sites were then used to make an in-frame *EYFP:LeNRT2.3* fusion construct. The final construct *35S:EYFP-LeNRT2.3*/pMON530 and the empty vector *35S:EGFP*/pMON530 were transiently expressed in onion epidermal cells using a particle gun-mediated system (PDS-1000/He; Bio-Rad). The bombarded cells were held in the dark at 22 °C for 12 h followed by YFP imaging using confocal microscopy (Carl Zeiss; LSM 510 Meta) with excitation at 513 nm. The transformed onion cells were plasmolyzed with 30% sucrose and viewed immediately.

2.6. Functional characterization of LeNRT2.3 in Xenopus laevis oocytes

The *LeNRT2.3* cDNA was subcloned as an *Xba*I-*Kpn*I fragment into the oocyte expression vector pOO2 [31], and cRNA was synthesized using the Ambion mMessage mMachine kit. *Xenopus* oocytes were isolated and maintained as described [24,32]. Oocytes were voltage clamped 1–2 d after injection essentially as described [32]. Voltage clamp recordings were initiated in a bath solution containing 230 mM mannitol, 0.15 mM $CaCl_2$, and 10 mM MES/Tris, pH5.5 [33]. High- and low-affinity nitrate uptake assays were performed as described [33]. Nitrate was added to the bath solutions as HNO_3 at the indicated concentrations.

2.7. Determination of NO_3^- or $^{15}NO_3^-$ levels

The *35S:LeNRT2.3* and its wild-type control were grown in hydroponic solution for 4 weeks as described above. The inflorescence stems were cut using a sharp razor, and xylem saps were collected for 6 h as described [34]. NO_3^- was extracted and determined by HPLC (Agilent 1200 series) using a PARTISIL 10 strong anion exchanger column (Whatman) as described [35].

For $^{15}NO_3^-$ Levels determination, plants were transferred to 0.1 mM $CaSO_4$ for 1 min, then to hydroponic solution containing 0.5, 5 or 20 mM $^{15}NO_3^-$ for 180 min, or 20 mM $^{15}NO_3^-$ for 5, 30 min. The roots were washed for 1 min in 0.1 mM $CaSO_4$ and separated from the shoots. The organs were dried at 80 °C for 48 h, weighed, and analyzed for total ^{15}N content using a continuous-flow isotope ratio mass spectrometer coupled with a carbon nitrogen elemental analyzer (Vario EL III/Isoprime) as described [36].

2.8. Statistical analysis

Two-tailed Student's *t* tests were performed. Differences were deemed significant at $P < 0.05$ and extremely significant at $P < 0.01$.

3. Results

3.1. LeNRT2.3 responses to nitrate in tomato

Nitrate is a key nutrient in plant growth and development. It is important to understand the mechanisms of nitrate transport in

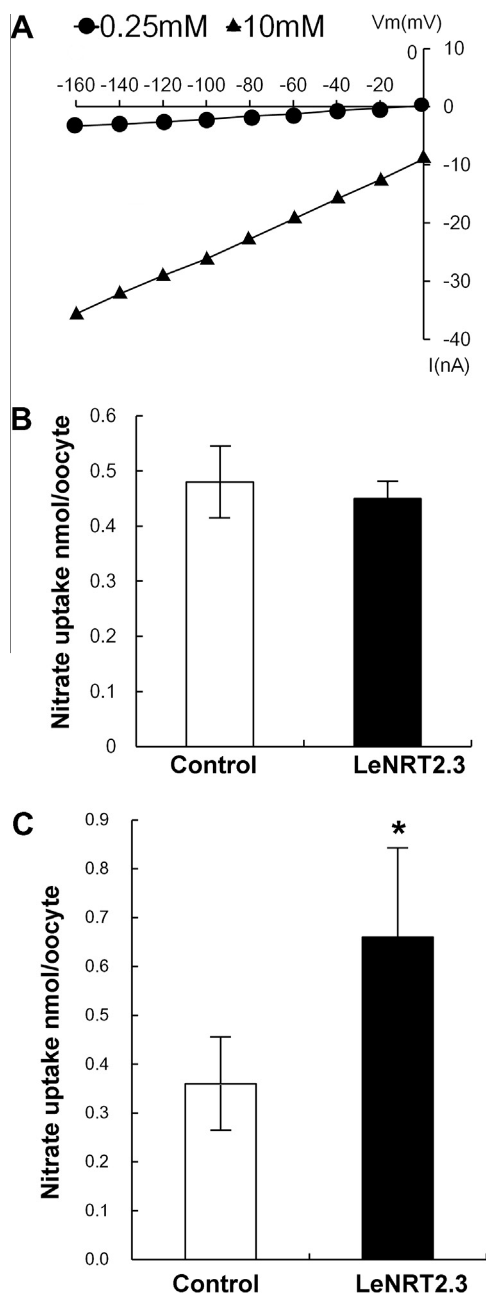

Fig. 4. LeNRT2.3 can transport nitrate. (A) Representative inward currents elicited by 0.25 (top) and 10 mM NO_3^- (bottom) in LeNRT2.3 cRNA injected oocytes. (B) and (C) High- and low-affinity nitrate uptake activity. Oocytes were incubated for 3 h with 0.25 mM (B) or 10 mM nitrate (C). $n = 4$ samples for both high- and low affinity uptake assays. Each sample consisted of four oocytes. $^*P < 0.05$.

plant. In order to identify nitrate transporters in tomato, we homo-cloned *LeNRT2.3* gene and analyzed the expression pattern of *LeNRT2.3* in response to nitrate. The protein sequence of LeNRT2.3 showed 77% identity with AtNRT2.4, 76% identity with AtNRT2.1 and 67% identity with AtNRT2.3 (Supplementary Fig. 1A). *LeNRT2.3* is predicted to contain three exons and two introns, and encodes a protein of 531 amino acids with twelve typical transmembrane domains (Supplementary Fig. 1B).

Quantitative realtime-RT-PCR was performed to characterize the expression pattern of *LeNRT2.3* under various nitrate treatment. *LeNRT2.3* expression was slightly induced by 0.5 mM NO_3^-, and strongly induced by 5 mM NO_3^- in the whole plants (Fig. 1A). In situ hybridization data further confirmed the result (Fig. 3B–D).

Quantitative realtime-RT-PCR analyses showed that *LeNRT2.3* is ubiquitously expressed in many tissues. The expression level is higher in flowers, leaves and roots than stems (Fig. 1B). These data suggest that *LeNRT2.3* may have functions in shoots and roots.

3.2. LeNRT2.3 is localized to the plasma membranes

To further understand the function of *LeNRT2.3*, *LeNRT2.3* was fused in frame with the enhanced yellow fluorescent protein (EYFP). Transient expression of EYFP:LeNRT2.3 in onion epidermal cells showed that LeNRT2.3 was localized to the plasma membranes (Fig. 2A and B) compared with the diffuse nucleo cytoplasmic localization of the EYFP control (Fig. 2C and D).

3.3. LeNRT2.3 is expressed in vascular rhizodermal and pericycle cells

To determine the cell-specific expression pattern of *LeNRT2.3*, in situ hybridization analysis was performed using the *LeNRT2.3* antisense probe. *LeNRT2.3* accumulated to rhizodermal and pericycle cells in roots induced by NO_3^- (Fig. 3B–D). In the control experiment using the sense *LeNRT2.3* probe, no signal was detected (Fig. 3A). These results indicated that *LeNRT2.3* was expressed in rhizodermal and pericycle cells in roots.

3.4. LeNRT2.3 functions in nitrate transport

To determine whether LeNRT2.3 is a nitrate transporter, *X. laevis* oocytes were injected with LeNRT2.3 to perform electrophysiological analyses. In injected oocytes, a large inward current was induced exposed to 10 mM nitrate by contrast to a small inward current exposed to 0.25 mM nitrate (Fig. 4A). Uptake analyses in oocytes showed that LeNRT2.3 mediated nitrate uptake at 10 mM nitrate (Fig. 4C), but not 0.25 mM nitrate (Fig. 4B).

Considering that LeNRT2.3 is expressed in the plasma membranes of rhizodermal and pericycle cells (Fig. 3B–D), we suggest that LeNRT2.3 might function to take up nitrate into roots and also load nitrate into xylems.

3.5. Overexpression of LeNRT2.3 increases nitrate concentrations in tomato

To investigate the in vivo function of *LeNRT2.3*, *35S:LeNRT2.3* construct was introduced into wild type Micro-Tom. Eight homozygous transgenic lines (1, 2, 3, 4, 5, 6, 7, 8#) were isolated and confirmed by kanamycin segregation. Quantitative realtime-RT-PCR analysis showed that *LeNRT2.3* mRNA levels were significantly enhanced in 1# and 2# (Fig. 5A) and in other 6 lines (Supplementary Fig. 2A).

To examine whether overexpression of LeNRT2.3 could affect nitrate uptake or transport in transgenic plants, 1-week-old plants were shifted to half-strength MS hydroponic medium for another 3 weeks. Then shoot and root tissues were harvested and their nitrate contents were determined by HPLC. As shown in Fig. 5B and Supplementary Fig. 2B, nitrate contents were both increased in shoots and roots in transgenic tomato compared with that in wild type.

To confirm that *LeNRT2.3* plays function in nitrate uptake and long-distance transport, short-term nitrate uptake and translocation were analyzed by exposing the plants to $^{15}NO_3^-$. The nitrate uptake activity of the transgenic plants was higher compared to that of the wild type when exposed to 0.5, 5 or 20 mM $^{15}NO_3^-$ for 180 min, more ^{15}N were translocated to the shoot in the transgenic plants compared to wild type (Fig. 5C). The result was consistent with that of nitrate content in xylem sap (Fig. 5E). The shoot/root ^{15}N concentration ratio was 0.22 in the transgenic plants, and 0.27 in the wild type labeling with ^{15}N for 5 min. After 30 min

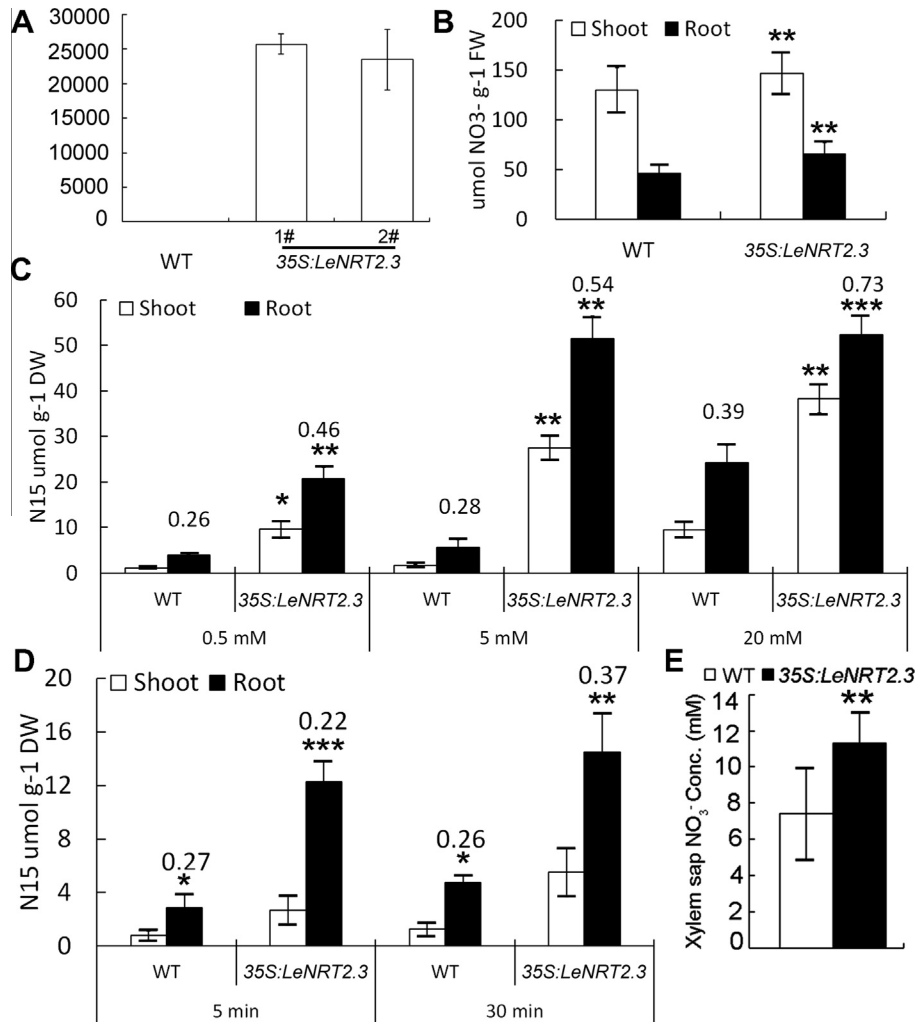

Fig. 5. The altered distribution of nitrate in *35S:LeNRT2.3* transgenic tomato. (A) The enhanced expression levels in *35S:LeNRT2.3*. *GAPDH* was used as an internal control. (B) Nitrate concentrations in wild-type (Micro-Tom) and *35S:LeNRT2.3* in hydroponic. $n = 30$ in shoots, and $n = 6$ in roots. (C) Root and shoot ^{15}N contents of wild-type (Micro-Tom) and *35S:LeNRT2.3* in hydroponic under nitrate treatment. The number above the bar is the shoot/root nitrate concentration ratio. (D) Nitrate uptake activity of wild-type (Micro-Tom) and *35S:LeNRT2.3* under nitrate treatment for the indicated time. The number above the bar is the shoot/root nitrate concentration ratio. (E) Nitrate concentration in the xylem sap. $n = 8$ in wild plants, and $n = 6$ in *35S:LeNRT2.3* plants. Values are mean ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

exposure, the shoot/root ratio was 0.37 in the transgenic plants, and 0.26 in the wild type (Fig. 5D). To confirm that *LeNRT2.3* plays function in xylem transport of nitrate, xylem exudates were analyzed. Nitrate concentration was higher in the xylem sap of the transgenic plants than that of the wild type (Fig. 5E; Supplementary Fig. 2C). These results suggested that LeNRT2.3 is involved in nitrate uptake and long-distance transport.

3.6. Overexpression of LeNRT2.3 increases biomass and fruit weight in tomato

N uptake and mobilization have effects on the biomass of plants [37]. To evaluate the function of LeNRT2.3, transgenic tomato biomass was compared with wild type plants under 0.5 or 5 mM NO_3^-. The shoot biomass was significantly increased in the *35S:LeNRT2.3* transgenic tomato under 0.5 or 5 mM NO_3^- (Fig. 6A; Supplementary Fig. 3A), and the root biomass was significantly increased in *35S:LeNRT2.3* tomato under 5 mM NO_3^- (Fig. 6B; Supplementary Fig. 3B). Fruit weight was also increased in *35S:LeNRT2.3* tomato under 5 mM NO_3^- (Fig. 6C; Supplementary Fig. 3C). The results suggest that *LeNRT2.3* is involved in biomass accumulation and fruit weight in tomato via regulation of nitrate transport.

4. Discussion

4.1. LeNRT2.3 is a nitrate transporter in tomato

In tomato, two NPF/NRT1 and three NRT2 genes are described inducible by nitrate [26–28]. Among the five genes, LeNRT2.3 likely encodes a low-affinity transporter for nitrate. LeNRT2.3 locates to the plasma membranes (Fig. 2A and B) and was inducible by nitrate in a dose dependent manner (Fig. 1A). In situ hybridization results showed that LeNRT2.3 expresses in the rhizodermal and pericycle cells in roots (Fig. 3B–D).

Analysis in *Xenopus* oocytes showed that LeNRT2.3 may act as a low-affinity nitrate transporter (Fig. 4). In injected oocytes, a large inward current was induced exposed to 10 mM nitrate by contrast to a small inward current exposed to 0.25 mM nitrate (Fig. 4A). Uptake analyses in oocytes showed that LeNRT2.3 mediated nitrate uptake at 10 mM nitrate (Fig. 4C), but not 0.25 mM nitrate (Fig. 4B). Furthermore, overexpression of *LeNRT2.3* increase nitrate uptake (Fig. 5C and D). Taking together, these results suggest that LeNRT2.3 functions as a low-affinity nitrate transporter.

In contrast, NRT2 members act as high-affinity transporters in other species such as *Arabidopsis* [38,39], rice [15], barley [17] or

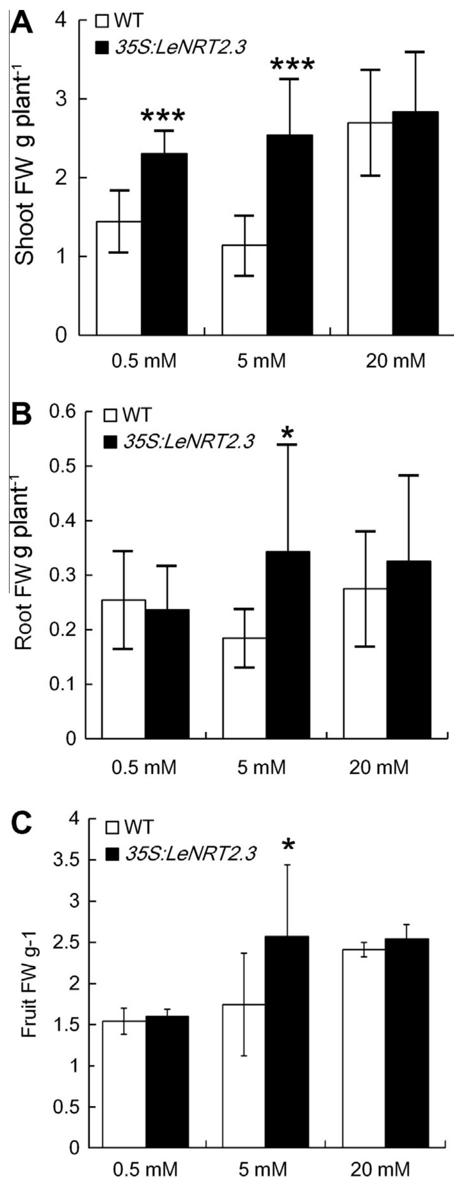

Fig. 6. Increased biomass and fruit weight in *35S:LeNRT2.3* in transgenic tomato. (A) Increased shoot mass in *35S:LeNRT2.3* with 0.5 and 5 mM nitrate. *n* = 8, 9, 12 in wild type plants with 0.5, 5, 20 mM NO_3^- treatment, and *n* = 12 in 35:LeNRT2.3 plants. (B) Increased root mass in *35S:LeNRT2.3* with 5 mM nitrate. *n* = 8, 9, 12 in wild type plants with 0.5, 5, 20 mM NO_3^- treatment, and *n* = 12, 8, 12 in 35:LeNRT2.3 plants. (C) Increased fruit weight in *35S:LeNRT2.3* with 5 mM nitrate. *n* = 8 in wild type plants with 0.5, 5, 20 mM NO_3^- treatment, and *n* = 9 in 35:LeNRT2.3 plants. Values are mean ± S.E. $^*P < 0.05$, $^{***}P < 0.001$.

Chlamydomonas [20]. NAR2 may also be required in high-affinity nitrate transport regulated by LeNRT2.3. NAR2 is involved in high-affinity nitrate uptake as a partner with NRT2 to establish HATS activity in several plant species [20,40–44]. HATS is reduced in *nar2.1* mutant in *Arabidopsis* [45]. LeNRT2.3 alone without NAR2 can mediate low-affinity nitrate transport in *Xenopus* oocytes (Fig. 4A and C). However, NAR2 may be required for the high-affinity nitrate transport activity of LeNRT2.3 as other NRT2 members. It is interesting to know if there is NAR2 protein forming component with LeNRT2.3 to change nitrate uptake rate in tomato. Further biochemical studies are required to calculate the exact K_m for LeNRT2.3 and different pH values may be helpful.

4.2. LeNRT2.3 may play a double role in roots and shoots as a nitrate transporter

Nitrate uptake and long-distance transport are two critical steps in regulating nitrogen availability for plant growth. Several genes are reported involved in nitrate transport in both shoots and roots in *Arabidopsis*. AtNRT2.4 plays roles in taking up nitrate in roots and loading into the phloem in shoots [12]. AtNPF6.3/AtNRT1.1 acts in nitrate uptake and root to shoot transport [7–9,40]. AtNPF7.3/AtNRT1.5 participates in nitrate influx/efflux and is expressed in shoots [23,43]. In rice, OsNPF2.4 plays roles in nitrate acquisition and long-distance transport [46]. OsNRT2.3a functions in long-distance nitrate transport from root to shoot but not uptake in roots [47]. However, much less is known about nitrate long-distance transport compared with uptake.

In tomato, LeNRT2.3 is suggested to play a double role in nitrate uptake in roots and long-distance transport from root to shoot. The phylogenetic analysis showed that LeNRT2.3 displayed high amino acid similarity compared with AtNRT2.4 (Supplementary Fig. 1A), indicating that LeNRT2.3 has a similar protein function with its homolog in *Arabidopsis*. The tissue localization of *LeNRT2.3* expression (Fig. 3B–D) and N^{15}/NO_3^- analyses (Fig. 5B–E, and Supplementary Fig. 2B and C) suggest that *LeNRT2.3* plays a key role in the xylem transport of nitrate from root to shoot and uptake in roots.

The increased nitrate concentrations in shoots (Fig. 5B and C) could be a direct consequence of uptake increase in roots. Wild type plants showed higher nitrate contents in shoots and roots at high nitrate solution (20 mM) compared with those at low nitrate solution (0.5 and 5 mM) (Fig. 5C). Transpiration may act to power the transport of nitrate via the xylem from the root to the shoot [48]. However, the ratios of shoot/root were higher in *35S:LeNRT2.3* compared with those in wild type plants at low (0.5 and 5 mM) or high nitrate solutions (20 mM) (Fig. 5C). The results showed that the overexpression of *LeNRT2.3* caused strong increased translocation of nitrate from root to shoot, and other mechanism such as transpiration may be the second cause.

However, we do not exclude the possibility that the enhancement of the nitrate content in shoot requires the up-regulation of the nitrate uptake. It may be that the accumulation of nitrate in root induces disturbance in the signal transduction pathway, and regulates the expression of other unidentified transporters by enhanced influx nitrate in roots together with LeNRT2.3.

4.3. LeNRT2.3 regulates nitrogen-based biomass

Nitrogen is a major limiting factor in plant growth and yield. Genes affect plant growth by nitrate uptake or remobilization in *Arabidopsis*. Double mutants of *nrt2.1 nrt2.2* and triple mutants of *nrt2.1*, *nrt2.2* and *nrt2.4* display less biomass under nitrogen limitation [12,49]. Loss of *AtNPF2.13/NRT1.7* causes growth retardation under nitrogen starvation [50]. *Atnpf7.2/nrt1.8* mutant shows growth reduction treated with NO_3^- and Cd^{2+} [24]. It is deduced that nitrate serves both as nutrient and signal on plant metabolism and growth [5,51–55]. The higher expression levels of *LeNRT2.3* in flowers and leaves indicate that *LeNRT2.3* plays a pivotal function in shoots development. The increased nitrate content in shoots of *35S:LeNRT2.3* may be a signal to enhance biomass in the transgenic plants. It will be interesting to see whether nitrate functions as a signal on plant metabolism and growth.

Disclosure summary

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.03.016.

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