NRT1.1B improves selenium concentrations in rice grains by facilitating selenomethionine translocation

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Summary
Selenium (Se) is an essential trace element for humans and other animals, yet approximately one billion people worldwide suffer from Se deficiency. Rice is a staple food for over half of the world’s population that is a major dietary source of Se. In paddy soils, rice roots mainly take up selenite, Se speciation analysis indicated that most of the selenite absorbed by rice is predominately transformed into selenomethionine (SeMet) and retained in roots. However, the mechanism by which SeMet is transported in plants remains largely unknown. In this study, SeMet uptake was found to be an energy-dependent symport process involving $H^+$ transport, with neutral amino acids strongly inhibiting SeMet uptake. We further revealed that NRT1.1B, a member of a rice peptide transporter (PTR) family which plays an important role in nitrate uptake and transport in rice, displays SeMet transport activity in yeast and Xenopus oocyte. The uptake rate of SeMet in the roots and its accumulation rate in the shoots of nrt1.1b mutant were significantly repressed. Conversely, the overexpression of NRT1.1B in rice significantly promoted SeMet translocation from roots to shoots, resulting in increased Se concentrations in shoots and rice grains. With vascular-specific expression of NRT1.1B, the grain Se concentration was 1.83-fold higher than that of wild type. These results strongly demonstrate that NRT1.1B holds great potential for the improvement of Se concentrations in grains by facilitating SeMet translocation, and the findings provide novel insight into breeding of Se-enriched rice varieties.

Keywords: NRT1.1B, selenomethionine, selenite, transport, rice (Oryza sativa L.).

Introduction
Selenium (Se) is an essential trace element for humans and other animals that is critical for antioxidation by forming the active site of glutathione peroxidases as selenocysteines (SeCys) (Schwarz and Foltz, 1958; Rotruck et al., 1973; Steinbrenner and Sies, 2009). Se is also associated with multiple health benefits, such as enhancing immunity, delaying AIDS progression in HIV-infected patients, reducing the incidence of cancers and maintaining male fertility (Clark et al., 1998; McKenzie et al., 1998; Foresta et al., 2002; Chantratita et al., 2004; Ryan-Harshman and Aldoori, 2005; Shin et al., 2007). Many of these functions are mediated by selenoproteins, which contain Se in the form of SeCys. Sufficient Se intake supports maximal expression of selenoproteins (Xia et al., 2005), whereas inadequate dietary Se intake is closely associated with endemic diseases, such as Keshan disease, and Kashin–Beck disease (Tan et al., 2002). A recommended dietary allowance for health benefits is 50–60 μg/day for males and females (Institute of Medicine, 2000). However, the recommended daily intake is not achieved in the majority of European countries and in some parts of China (Rayman, 2004). Indeed, the majority of the world’s population consumes less Se than the optimal amounts required for protection against cancer, cardiovascular diseases and other severe infectious diseases, and it is estimated that approximately one billion people worldwide suffer from Se deficiency (Combs, 2001; Haug et al., 2007).

Human Se is primarily acquired from plant foods, especially cereals, although milk, egg, meat and fish are also potential sources of Se (Rayman, 2012). Rice is a staple food for over half of the world’s population. However, a survey of global rice samples revealed that approximately 75% of grains likely fail to provide 70% of the daily recommended Se intake (Williams et al., 2009). Many factors affect Se concentration in grains, such as Se levels in soil, varietal differences, agronomic measures and the use of Se fertilizers (Eurola et al., 1991; Hartikainen, 2005; Hawkesford and Zhao, 2007; Lyons et al., 2005). Under soil Se-deficient conditions, the use of Se fertilizers is often the only option to increase Se concentrations, and foliar spraying with Se fertilizers or supplying soils with fertilizers with Se can effectively increase Se concentration in grains (Deng et al., 2017; Eurola et al., 1991; Hartikainen, 2005; Li et al., 2010). However, foliar application of Se usually results in an uneven Se concentration in grains and increases the cost of Se-enriched rice production; this technique is also difficult to perform under rainy or windy conditions. Thus, soil amendment of Se fertilizers is suggested as a feasible approach to increase grain Se concentration. However, Se added to soils is not efficiently taken up by plants. For example the total recovery (grain and straw) of applied Se was found to be only 20%–35%. The residual Se might be leached, volatilized by soil microbes, or retained in the soil as unavailable forms to plants (Broadley et al., 2010). In contrast, genetic biofortification, such as breeding Se-enriched cultivars with high grain Se
concentrations, provides a promising cost-effective and sustainable approach to improve grain Se concentrations by enhancing the utilization efficiency of Se in soils.

Among the factors controlling Se accumulation in grains, Se uptake and transport are the most fundamental physiological processes. Selenate and selenite are the predominant forms of Se available to plants in soils. Because selenate is readily reduced to selenite under flooding conditions, rice plants growing in paddy soils mainly absorb selenite. Selenite naturally exists in diverse forms, such as H₂SeO₃, H₂SeO₄⁻, and SeO₃²⁻, in solutions (Lauchli, 1993), and H₂SeO₄⁻ and HSeO₃⁻ are taken up by rice roots through aquaporins and the phosphate transporter OsPT2 respectively (Li et al., 2008; Zhang et al., 2006a, 2014; Zhao et al., 2010). Although OsPT2-overexpressing plants display a significantly enhanced root selenite uptake rate, the selenite absorbed is poorly translocated to shoots, thereby limiting the increase in Se concentration in grains (Zhang et al., 2014). Previous study suggested that a large proportion of the selenite absorbed by plant roots is transformed into organic Se compounds, such as SeMet (Kahakachchi et al., 2004; Li et al., 2008). Therefore, enhancing the efficiency of root-to-shoot SeMet translocation may increase grain Se concentration. However, the mechanism of SeMet transport in plants remains unclear. Plant peptide transporters (PTRs) transport a broad spectrum of substrates, such as nitrate, peptides, amino acids, dicarboxylates, glucosinolates, IAA and ABA (Ler et al., 2014). NRT1.1B, a member of the PTR family, encodes a protein containing a peptide-transporter domain. We showed previously that NRT1.1B, predominantly expressed in the vascular tissues of roots, leaf sheaths, leaf blades and culms. Together with its plasma membrane localization, NRT1.1B was demonstrated to be involved in root-to-shoot nitrate transport (Hu et al., 2015). Here, we further reveal that NRT1.1B also mediates the root-to-shoot translocation of SeMet in rice. NRT1.1B overexpression significantly improved Se concentrations not only in shoots but also in grains. Our findings provide novel insights into breeding Se-enriched rice varieties by facilitating SeMet translocation.

Results

SeMet is the dominant Se form in rice roots

As selenite is the dominant form of Se in paddy soil, we first examined Se forms in different organs of rice seedlings supplied with selenite for 3 days. Enzyme-digested extracts of the roots, leaf sheaths and leaf blades were subjected to Se speciation analysis. The chromatogram of a mixed Se standard solution from HPLC-ICP-MS was provided as a reference (Figure S1). In the root extracts, selenite, SeMet, selenocystine (SeCys2), methylselenocysteine (MeSeCys) and unidentified Se forms were detected (Figure 1a). SeMet was the major Se form that constituted 46% of the total Se, followed by selenite and SeCys2, which accounted for 30% and 15% respectively; only trace amounts of MeSeCys (4%) and unidentified Se forms (6%) were detected. In the extracts of leaf sheaths and leaf blades, SeMet, SeCys2 and MeSeCys were found but not selenite (Figure 1a). SeMet was the dominant Se form, corresponding to 83% and 85% in leaf sheaths and leaf blades, respectively, and SeCys2 and MeSeCys accounted for 7% and 10% in leaf sheaths and 7% and 7% in leaf blades respectively.

After selenite treatment for 3 days, the rice seedlings were transferred back to nutrient solutions without Se for another 3 days, and we further investigated changes in the concentrations of various Se forms in different organs. Selenite and SeMet concentrations in the roots decreased by 2.24- and 1.98-fold, respectively (Figure 1a,b), whereas the SeMet concentrations in leaf sheaths and leaf blades increased by 1.39- and 2.44-fold respectively (Figure 1a,b). SeMet accounted for 91% and 96% in leaf sheaths and leaf blades respectively. These results revealed that most of the absorbed selenite was transformed to SeMet in the roots and then transported to the shoots.

Additionally, the root SeMet concentrations were 10.38- and 31.58-fold higher than those in the leaf sheaths and leaf blades, respectively, after 3 days of selenite treatment, and the SeMet concentrations in the roots was 3.78- and 6.53-fold higher than those in the leaf sheaths and leaf blades, respectively, when the seedlings were transferred back to nutrient solutions without Se for another 3 days (Figure 1b). This finding suggested that a large quantity of SeMet was retained in the roots and was not transported to the shoots.

SeMet uptake is an energy-dependent symport process

To investigate whether SeMet is taken up via carriers, we performed concentration- and time-dependent kinetic experiments of SeMet uptake with the rice seedlings. The results showed that the SeMet uptake rate increased markedly as the exogenous SeMet levels increased to 3.2 μM, then increased slowly and reached a plateau at high SeMet levels (Figure 2a). The SeMet uptake rate fit a Michaelis– Menten-type correlation with the SeMet level. Vmax and Km were 132.30 ± 7.51 mg/kg dry weight (DW) per h and 8.01 ± 1.05 μmol/kg DW, respectively, with R² of 0.99. Similarly, the Se concentration in the roots increased sharply over time and gradually reached a plateau at 4 h when the seedlings were supplied with SeMet (Figure 2b). This result indicated that SeMet uptake by rice roots exhibited the characteristic of saturation kinetics with time-points. Thus, SeMet uptake was postulated to be a carrier-mediated process supported by concentration- and time-dependent saturation kinetics.

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2, 4-dinitrophenyl (DNP) are typical respiratory inhibitors that collapse the proton-motive force by allowing protons to freely transverse membranes. To determine whether SeMet uptake is an energy-dependent symport process involving H⁺ transport, the effects of CCCP and DNP on SeMet uptake were investigated. The results indicated that CCCP and DNP decreased the Se concentration in roots by 94.5% and 97.0%, respectively, suggesting that CCCP and DNP largely inhibit SeMet uptake (Figure 2c). Therefore, SeMet uptake is an energy-dependent symport process involving H⁺ transport.

SeMet uptake is strongly inhibited by neutral amino acids

It has been postulated that SeMet, an analogue of Met, shares common transporters with other amino acids. To assess this hypothesis, we investigated the effects of amino acids on SeMet uptake by rice roots. Competitor amino acids were maintained at low concentrations (0.2 mM) to ensure that transport systems were active. It was found that most of the amino acids tested, except for the basic amino acids, lysine (Lys), arginine (Arg) and histidine (His), significantly inhibited SeMet uptake (Figure 2d). Neutral amino acids, namely, methionine (Met), tyrosine (Tyr), phenylalanine (Phe) and leucine (Leu), elicited the strongest inhibitory effects on SeMet uptake, by 74%, 71%, 69% and 67%, followed by serine (Ser), alanine (Ala), valine (Val), proline (Pro), threonine (Thr), cysteine (Cys), isoleucine (Ile), glutamine...
(Gln) and glycine (Gly) which inhibited SeMet uptake by 51%, 49%, 48%, 47%, 44%, 44%, 41%, 41% and 40% respectively. Tryptophan (Trp), asparagine (Asn) and the acidic amino acids, glutamic acid (Glu) and aspartic acid (Asp), elicited relatively slight inhibitory effects of 38%, 24%, 30% and 23% respectively. These results suggest that the carrier proteins

Figure 1 Assays of Se speciation in rice seedlings supplied with selenite. (a) Chromatograms of Se speciation based on HPLC-ICP-MS. (b) Se concentrations of different Se species in roots, leaf sheaths and leaf blades when supplied with selenite for 3 days and then cultured for another 3 days without Se. Values are the means ± SD (n = 3).

Figure 2 Assays of physiological characteristics of SeMet uptake by rice seedlings. (a) Concentration- and (b) time-dependent SeMet uptake kinetics. (c) Effects of respiration inhibitors on SeMet uptake. (d) Competition assay of SeMet uptake using different amino acids. Values are the means ± SD (n = 3). Asterisks indicate significant differences between control and treatments as evaluated by Student’s t-tests: *P < 0.05 and **P < 0.01.
responsible for the transport of neutral amino acids also mediate SeMet transport.

**NRT1.1B displays SeMet transport activity in vitro**

To investigate whether NRT1.1B is involved in SeMet transport, SeMet transport activity of NRT1.1B was examined in vitro. Yeast strains expressing a full-length NRT1.1B cDNA were constructed and incubated in liquid medium containing SeMet. According to the results of qRT-PCR, expression of NRT1.1B was substantially increased in the NRT1.1B-transgenic strain relative to the control strain carrying empty vector (Figure 3a). The SeMet transport rate in the NRT1.1B-transgenic strain was significantly higher than that in the control strain (Figure 3b). The SeMet transport activity of NRT1.1B was also evaluated in Xenopus oocytes, and uptake measurement showed that the SeMet transport rate in the oocytes injected with NRT1.1B cRNA was significantly higher than that in the oocytes injected with water (Figure 3c). These results strongly demonstrated that NRT1.1B has a transport activity for SeMet.

**nrt1.1b mutant displays defects in SeMet uptake and transport**

To determine the potential function of NRT1.1B in SeMet uptake and transport in rice plant, we further characterized its loss-of-function mutant nrt1.1b (Hu et al., 2015). Concentration-dependent SeMet uptake kinetics showed that with increasing exogenous SeMet concentrations, the uptake rate of SeMet in wild type and nrt1.1b mutant fit well into a Michaelis–Menten-type correlation (Figure 4a). The uptake rate of SeMet in wild type was significantly higher than that in nrt1.1b mutant at different SeMet levels. \( V_{\text{max}} \) and \( K_m \) were 148.42 ± 7.55 mg/kg DW/h and 11.69 ± 1.16 \( \mu \)mol/kg DW for wild type, respectively, and 119.20 ± 7.55 mg/kg DW/h and 11.85 ± 1.77 \( \mu \)mol/kg DW for nrt1.1b mutant, respectively. \( R^2 \) was 0.99 and 0.98 for wild type and nrt1.1b, respectively. Although wild type and nrt1.1b mutant displayed the same affinity for SeMet, the \( V_{\text{max}} \) of wild type was significantly higher than that of nrt1.1b, indicating that the mutation of NRT1.1B may cause a decrease in \( V_{\text{max}} \) and consequently result in a defect in SeMet uptake.

Concentration-dependent SeMet accumulation kinetics revealed that the Se accumulation rate in the shoots of wild type and nrt1.1b increased linearly, with linear correlations with exogenous SeMet concentrations (Figure 4b). For wild type, the linear regression equation and \( R^2 \) were \( Y = 93.27X + 213.14 \) and 0.98 respectively; for nrt1.1b, the results were \( Y = 68.52X + 127.91 \) and 0.99 respectively. As the Se accumulation rates within 2 h in the shoots of the wild type were significantly higher than those of the nrt1.1b mutant at different SeMet levels, NRT1.1B possibly mediates root-to-shoot transport of SeMet.

**NRT1.1B overexpression increases root-to-shoot translocation of SeMet**

To investigate whether NRT1.1B increases SeMet translocation from roots to shoots, we performed SeMet transport assays in wild type (Nip) and NRT1.1B-overexpressing plants exposed to SeMet. Two lines generated using the rice ACTIN1 promoter, OE-31 and OE-72, with significantly increased expression of NRT1.1B were selected for further study (Figure 5a). SeMet concentration in xylem sap was used as an indicator of the capacity of SeMet translocation from roots to shoots, and the results showed that the SeMet concentration in xylem sap collected from OE-31 and OE-72 was 1.50- and 1.65-fold higher, respectively, than that from the wild-type plants (Figure 5a). In addition, the ratio of SeMet content between the shoot and root could represent the activity of long-distance SeMet transport (Zayed et al., 1998), and our results showed that the ratios of SeMet content of leaf blade/root, leaf sheath/root, and shoot/root OE-31 and OE-72 were significantly higher than those of wild type (Figure S3a). Thus, overexpression of NRT1.1B significantly enhanced SeMet translocation from roots to shoots. Moreover, compared with wild type, the SeMet concentration in the leaf sheaths and leaf blades was increased by 1.35- and 1.43-fold, respectively, for OE-31 and by 1.53- and 1.61-fold, respectively, for OE-72 (Figure 5b). These findings suggest that the increased activity of SeMet transport is critical for improving the Se accumulation in shoots.

The rice ACTIN1 promoter used to generate overexpressing lines OE-31 and OE-72 is constitutively expressed in most tissues. However, this expression pattern might not be optimal for enhancing SeMet transport compared with that mainly expressed in the vascular tissues. Our previous study indicated that the gcdsP promoter is specifically expressed in vascular tissues, with most abundant expression in the roots, followed by the spike stalks, stems, leaf blades and leaf sheaths (Chen et al., 2001). We thereby generated NRT1.1B-overexpressing plants driven by gcdsP, and two lines, OEvp-21 and OEvp-43, with significantly increased expression of NRT1.1B were selected for further study (Figure S2a). We found the SeMet concentration in xylem sap collected from OEvp-21 and OEvp-43 was 2.05- and 2.23-fold higher than that in wild-type plants (ZH11) respectively (Figure 5c). In addition, the leaf blade/root, leaf sheath/root, and shoot/root SeMet content ratios of OEvp-21 and OEvp-43 were also significantly higher than those of wild type (ZH11) (Figure S3b). Compared with wild-type plants (ZH11), the SeMet concentrations in leaf sheath and leaf blade were increased by 1.49- and 1.63-fold, respectively, for OEvp-21 and by 1.57- and

![Figure 3](image-url) **Figure 3** SeMet transport activity assays in yeast and oocyte. (a) Relative expression of NRT1.1B in yeast transformed with pYES2 empty vector and pYES2-NRT1.1B. (b) SeMet transport rate in yeast transformed with pYES2 empty vector and pYES2-NRT1.1B. (c) SeMet transport rate in the oocyte injected with NRT1.1B cRNA compared with that in the oocyte injected with water. Values are the means ± SD (n = 5 for yeast and n = 8 for oocyte). Asterisks indicate significant differences between pYES2 yeasts and pYES2-NRT1.1B yeasts or between water and NRT1.1B cRNA injected oocytes as evaluated by Student’s t-tests: \( *P < 0.05 \) and \( **P < 0.01 \).
when supplied with selenite ZH11 1.24- and 1.21-fold, respectively, and those of OE-72 were 1.19-, and leaf blades of OE-31 were significantly increased by 1.24-, selenite. As expected, Se concentrations in the roots, leaf sheaths and leaf blades of OEvp-21 were increased by 1.35-, 1.25- and 1.32-fold, and those of OEvp-43 were increased by 1.53-, 1.31- and 1.37-fold (Figure 6b). These results collectively suggested that NRT1.1B overexpression facilitates more Se translocation from roots to shoots, thereby resulting in increased Se concentrations in leaf sheaths and leaf blades of rice seedlings.

Previous study revealed that OsPT2 and OsNip2;1 are involved in selenite uptake in rice plant (Zhang et al., 2014; Zhao et al., 2010), thus we examined the expression levels of OsPT2 and OsNip2;1 in the roots of NRT1.1B-overexpressing lines. Interestingly, expression of OsPT2 and OsNip2;1 was significantly increased in the roots of OE-31 and OE-72, and that of OsPT2 was also significantly increased in the roots of OEvp-21 and OEvp-43, which may also promote selenite uptake in paddy soils (Figure S4a,b).

NRT1.1B overexpression improves Se concentrations in rice grains
To further investigate whether NRT1.1B increases Se concentrations in rice grains, we examined Se concentrations in the grains of wild type and NRT1.1B-overexpressing plants grown in the field. Expression of NRT1.1B was significantly increased by 29.73- and 39.30-fold in the shoots of OE-31 and OE-72, respectively, compared with that of wild type (Nip), and it was increased by 1.82- and 7.88-fold in the shoots of OEvp-21 or OEvp-43, respectively, compared with that of wild type (ZH11) (Figure S2b). As expected, Se concentrations were significantly increased in the grains of the overexpressing lines, reaching 1.25- and 1.43-fold in OE-31 and OE-72, respectively, compared with wild type (Nip) (Figure 6c). Notably, the grain Se concentrations of OEvp-21 and OEvp-43 were increased by 1.53- and 1.83-fold compared with wild type (ZH11) (Figure 6d), even though the increase in NRT1.1B expression in OEvp-21 and OEvp-43 was much lower than that in OE-31 and OE-72 (Figure S2b), displaying a more significant effect in improving grain Se concentrations. In addition, the plant yield of OEvp-21 and OEvp-43 was a little higher than that of wild type, though the difference was not significant (Figure S5a). The grain weight also exhibited no significant difference between overexpressing line OEvp-21 or OEvp-43 and wild type (Figure S5b). These results indicate that NRT1.1B holds great potential to increase grain Se concentrations with no repercussions to grain yield.

Discussion
Se naturally occurs in soils as selenide, elemental Se, thiosele-nate, selenite and selenate (Läuchli, 1993). Selenite and selenite
are the two dominant forms of Se available to plants, and redox potential and pH can greatly affect the forms of Se present in soils. Thermodynamic calculations show that selenate should be the predominant form in alkaline and well-oxidized soils (pE + pH > 15), whereas selenite is the form in reduced soils with pH from acidic to neutral (7.5 < pE + pH < 15) (Eirashidi et al., 1987; Li et al., 2008). Thus, selenite is the dominant Se form available to rice roots in paddy soils under flooding conditions. Our Se speciation analysis revealed that selenite was only present in roots and not in shoots (Figure 1), indicating that the selenite absorbed by rice roots was predominantly transformed into SeMet and was further translocated to shoots in the form of organic Se. We showed previously that selenite can be taken up through OsPT2, a phosphate transporter responsible for transporting inorganic phosphate (Pi) from roots to shoots (Ai et al., 2009; Zhang et al., 2014). However, a perplexing issue was encountered in the present study, namely, why selenite in roots was not directly transported to shoots via OsPT2. Actually, selenite is non-enzymatically quickly reduced to GSH-SeO$_3^-$ and GS-Se-GS in the cytoplasm and then reduced to GSH-Se$^-$ once it enters root epidermal cells (Anderson, 1993; Anderson and Scarf, 1983). GSH-Se$^-$ reacts with O-acetyl serine to form SeCys in plastids (Ng and Anderson, 1978a,b). Most of SeCys is transformed into SeMet and a small portion of SeCys is converted into MeSeCys (Neuhierl and Bock, 1996). Selenite metabolism in rice roots was further demonstrated via detection of a large quantity of SeMet and a trace amount of SeCys2 and MeSeCys in roots. This finding further revealed that most selenite was transformed into organic Se, resulting in a high concentration ratio of Pi to selenite in the cytoplasm of root cells. Moreover, OsPT2 is postulated to have a much higher affinity for Pi than for selenite. Thus, selenite was not found to be translocated from roots to shoots via OsPT2.

When rice seedlings were supplied with selenite, SeMet was the predominant form of Se in roots (Figure 1a). A previous study has indicated that the ratio of shoot Se to root Se ranges from 0.6 to 1.0 for plants supplied with SeMet, thereby suggesting that SeMet is not efficiently transported from roots to shoots (Zayed et al., 1998). In the present work, SeMet concentrations in roots of wild type (Nip) were 1.74-fold higher than those in leaf sheaths and leaf blades, respectively, when wild-type seedlings were supplied with SeMet for 3 h and then transferred back to nutrient solutions without Se for 3 days (Figure 5b). These results further demonstrated that a large quantity of SeMet was not transported to shoots but instead was retained in the roots. This finding may explain why Se was not readily translocated from roots to shoots when seedlings were supplied with selenite because a large proportion of absorbed selenite was predominantly transformed to SeMet in the roots. Thus, improving the capacity of SeMet transport from roots to shoots is a key step for enhancing Se transport when rice seedlings are supplied with selenite.

Concentration- and time-dependent uptake kinetics indicated that SeMet uptake exhibited saturation characteristics (Figure 2a, b). Moreover, SeMet uptake was largely inhibited by respiration inhibitors, such as CCCP and DNP (Figure 2c), a finding that is also supported by a previous study (Abrams et al., 1990). Therefore, SeMet uptake is an active process requiring a selective binding site and metabolic energy as a driving force. In addition, we found that SeMet uptake was remarkably inhibited by neutral amino acids, such as Met, Tyr, Phe, Leu, Ser, Ala, Val, Pro, Thr, Cys and Gin (Figure 2d), suggesting that SeMet shares common transporters with neutral amino acids. The molecular functions of several amino acid transporters (AATs) are characterized in Arabidopsis and rice. For instance AtAATs transport neutral and charged amino acids (Fischer et al., 2002; Lee et al., 2007), and
Peptide transporters can also transport amino acids in addition to a broad spectrum of other substrates such as nitrate, peptides, dicarboxylates, glucosinolates, IAA and ABA in plants (Leran et al., 2014). For example BnNRT1;2 exhibits a similar transport activity for nitrate and His (Zhou et al., 1998). AtpTR1 is a high-affinity peptide transporter that can also transport His (Dietrich et al., 2004). Because peptide transporters such as BnNRT1;2 and AtpTR1 exhibit transport activity for only a few amino acids, such as His (Dietrich et al., 2004; Zhou et al., 1998), these transporters have been postulated to have a higher selectivity for transporting amino acids than do AATs. Our previous study showed that NRT1.1B, a member of the rice PTR family, is involved in root-to-shoot nitrate translocation (Hu et al., 2015). Therefore, whether NRT1.1B also functions in the transport of SeMet is an extremely intriguing question. As expected, NRT1.1B showed transport activity for SeMet, as demonstrated in yeast and in oocytes. Moreover, the SeMet uptake rate of nrt1.1b mutant was significantly lower than that of wild-type rice. Further studies revealed that overexpression of NRT1.1B in rice significantly increased SeMet concentration in the xylem sap and in shoots when plants were supplied with SeMet (Figure 5a–d). In addition, the ratio of the SeMet content between shoots and roots in NRT1.1B-overexpressing lines was significantly higher than that in wild type (Figure S3a,b). These findings collectively confirm that NRT1.1B possesses SeMet transport activity and can mediate SeMet translocation from roots to shoots in rice. NRT1.1B is predominantly expressed in the vascular tissues of root, leaf sheath, leaf blade and culm (Hu et al., 2015), further supporting its role in transporting SeMet from roots to shoots.

Met, an analogue of SeMet, can inhibit SeMet uptake by 74%, suggesting that Met shares common transporters with SeMet (Figure 2d). Thus, overexpression of NRT1.1B may also stimulate Met translocation from roots to shoots. However, peptide transporters have a higher selectivity for transporting amino acids compared with AATs (Dietrich et al., 2004; Zhou et al., 1998). Thus, even though other neutral amino acids such as Tyr, Phe and Leu elicit the strongest inhibitory effects on SeMet uptake, but further investigation is required to determine whether overexpression of NRT1.1B may increase the translocation of these amino acids. Additionally, although SeMet and Met are common substrates of NRT1.1B, SeMet is significantly different from Met in both physical and chemical characteristics. It is therefore imaginable that SeMet and Met may use different binding sites of NRT1.1B. A previous study found that nitrate up-regulated expression of NRT1.1B (Hu et al., 2015), thus, SeMet uptake and transport may be enhanced by nitrate through induction of NRT1.1B expression.

In vascular tissues of rice leaf blades, xylem and phloem occur in close proximity to each other, and the metabolome sieve tubes in rice leaf veins are either in direct contact with or in close proximity to metaxylem vessels (Botha et al., 2008). Thus, SeMet exchange between xylem and phloem may occur after SeMet is translocated from the roots to leaf blades. Once SeMet reaches the xylem parenchyma, it may be transported through the phloem sieve tube complexes. Because NRT1.1B is highly expressed in leaf blade vascular tissues (Hu et al., 2015), SeMet can be transferred by NRT1.1B from the xylem to the phloem in leaf veins to ensure efficient remobilization of SeMet to seeds.

Our previous study found that Se concentrations in rice grains correlated positively with those in shoots (Zhang et al., 2006b). Because overexpression of NRT1.1B enhanced Se concentrations in shoots by increasing SeMet translocation from roots to shoots when supplied with selenite (Figure 6a,b), it was postulated that overexpression of this gene may also increase Se concentrations in grains. As expected, Se concentrations in the grains of the NRT1.1B-overexpressing transgenic lines OE-31 and OE-72 were significantly increased compared with those in wild type (Nip) (Figure 6c). Interestingly, although vascular-specific overexpression of NRT1.1B did not significantly increase grain yield, the Se concentration in the grains of OEvP-43 was increased up to 1.83-fold than that of wild-type plants (ZH11), providing a refined strategy for manipulating NRT1.1B expression to improve Se accumulation in grains (Figure 6d). SeMet has been reported as the main form of organic Se in rice grains (Fang et al., 2009; Li et al., 2010; Sun et al., 2010; Zhao et al., 2011), and as such, overexpression of NRT1.1B may increase SeMet translocation to grains. These results strongly demonstrated that overexpression of NRT1.1B could improve Se concentrations in grains by facilitating SeMet translocation. This study not only enhances our understanding of Se translocation from roots to shoots in rice, but also provides novel insight into breeding Se-enriched rice varieties by facilitating SeMet translocation.

**Experimental procedures**

**Plant materials and growth conditions**

Rice (Oryza sativa L.) wild-type Zhonghua 11 (ZH11), its nrt1.1b mutant and vascular-specifically NRT1.1B-overexpressing lines OEvP-21 and OEvP-43, wild-type Nipponbare (Nip) and its NRT1.1B-overexpressing lines OE-31 and OE-72 were used in this study. The NRT1.1B-overexpressing lines OE-31 and OE-72 were generated using rice ACTIN1 promoter, which is constitutively expressed in most tissues. The NRT1.1B-overexpressing lines OEvP-21 and OEvP-43 were generated using gdsP, a vascular-specific promoter isolated from Faveria anomala (Chen et al., 2001). The nrt1.1b mutant carries a T-DNA insertion in the intron and has defects in both nitrate uptake and nitrate root-to-shoot transportation (Hu et al., 2015). Seeds were surface-sterilized in 1% NaClO solution for 10 min, and then germinated in an incubator at 35°C. The young seedlings were transferred to Kimura B nutrient solution. The nutrient solutions were renewed every 3 days. pH was adjusted to 5.5 every day. The seedlings were cultured in a controlled growth chamber with a diurnal cycle of 14 h light at 25 °C and 10 h dark at 18 °C. The light intensity
was 300 μmol-photons/m²/s. The air humidity was controlled at 67%. Rice seedlings were continuously cultured for 3-4 weeks for experiments (Zhang et al., 2014).

**Enzyme hydrolysis**

Ground samples (0.1 g) were placed into 10 mL centrifuge tubes, and protease K (20 mg), lipase VII (10 mg) and pH 7.5 Tris-HCl were added up to a final volume of 5 mL. The tubes were gently shaken at 37 °C with 60 r.p.m. for 22 h in darkness, and then centrifuged at 1800 g for 30 min. The supernatants were collected, filtered through 0.45 μm filter and stored at −20 °C for Se speciation analysis (Sun et al., 2010).

**Se speciation analysis of rice seedlings cultured in selenite solution**

About 18 days of rice seedlings were transferred to Kimura B nutrient solution containing 2 μM Na₂SeO₃, and the rice seedlings were harvested for Se speciation analysis after 3 days. The other seedlings were transferred back to nutrient solutions without Se for another 3 days before being harvested for Se speciation analysis to investigate changes in the concentrations of Se forms. High-performance liquid chromatography (HPLC, Agilent Technologies LC1100 series) coupled with inductively coupled plasma mass spectrometry (ICP-MS, 7500ce, Agilent Technologies) was used for the Se speciation analysis. The different Se species were separated by an anion-exchange column (PRP X-100, 4.1 mm×250 mm, 10 μm) and a pre-column (Dionex AG14). The injection volume of individual sample was 50 μL. The mobile phase contained 5 μM ammonium citrate and 2% methanol (pH 4.3) at a flow rate of 1 mL/min was used. The ICP-MS operating conditions were as follows: RF power, 1400 W; sample depth, 8 mm from the load coil; Ar auxiliary gas flow rate, 0.8 L/min; spray chamber temperature, 2 °C. Peaks were identified by comparison with the retention times of the standard compounds. The Se standard sample was a mixture of 50 μL selenite, SeCys and SeMet and MeSeCys, respectively, which were purchased from Sigma. Se species were identified by the retention times and quantified by peak area measurements of the chromatographic signals by monitoring the isotope 82Se (Sun et al., 2010).

**Kinetics assay of SeMet uptake and accumulation**

The roots of the rice seedlings (ZH11 and its nrt1.1b mutant) were transferred to absorption solution containing 5 mM 2-morpholinoethanesulphonic acid (MES), 0.5 mM Ca(NO₃)₂ and SeMet at concentrations of 0, 0.4, 0.8, 1.6, 3.2, 6.4, 9.6, 12.8 or 19.2 μM (pH 5.0) for 2 h. After the termination of SeMet uptake, the roots were rinsed to remove the Se adsorbed onto the root surfaces. The roots and shoots were then separated for Se concentration analysis. Similarly, the roots of rice seedlings (ZH11) were placed in absorption solution containing 5 mM MES, 0.5 mM Ca(NO₃)₂ and 2 μM SeMet for 1, 2, 3, 4, 5, 6, 7 or 8 h. The roots were separated for Se concentration analysis (Zhang et al., 2014).

**Assay of SeMet uptake affected by respiration inhibitors**

The roots of rice seedlings were placed in absorption solution containing 5 mM MES, 0.5 mM Ca(NO₃)₂, 2 μM SeMet with and without 1 μM CCCP or 20 μM DNP, respectively, for 2 h. After the termination of SeMet uptake, the roots were rinsed and excised from the base of the shoots for Se concentration analysis (Zhang et al., 2014).

**Competitive assay of SeMet uptake with amino acids**

The roots of rice seedlings were placed in absorption solution containing 5 mM MES, 0.5 mM Ca(NO₃)₂ and 1 μM SeMet with 0.2 mM amino acid, including Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Tyr, Ser, Thr, Cys, Asn, Glu, Gly, Glu, Lys, Arg or His, respectively, for 2 h. After the termination of SeMet uptake, the roots were rinsed and excised from the base of the shoots for Se concentration analysis.

**Determination of Se concentration**

Dried and homogenized samples were weighed and put into 100 mL digestion tubes. Then 5 mL of an acid mixture of 4 mL HNO₃ and 1 mL HClO₄ were added. The samples were pre-digested overnight at room temperature, and then digested at 150 °C completely in a digestion oven. The digests were diluted with millipore water to a final volume of 25 mL. Total Se in the digested samples was determined by ICP-MS (Zhang et al., 2014).

**Construction of rice plants overexpressing NRT1.1B**

The CDS of NRT1.1B (japonica) was amplified and cloned into the binary vector pcAMBI2300-CamV 35S or pcAMBI2300-gdcsP between restriction sites Smal and XbaI to generate pcAMBI2300-35S: NRT1.1B or pcAMBI2300-gdcsP: NR T1.1B-overexpressing vectors respectively. The resulting vectors and the empty vectors were introduced into Agrobacterium strain AG1L respectively. Wild type (Nip or ZH11) were used as the recipients for Agrobacterium-mediated transformation to generate the transgenic rice as described previously (Liu et al., 2007). To generation of transgenic plants were further identified using PCR amplification of NPTII with the genome DNA. The relative expression of NRT1.1B was determined using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in NRT1.1B-overexpressing lines and wild type (Hu et al., 2015).

**SeMet uptake assay in yeast**

The full-length NRT1.1B cDNA was subcloned into pYES2 between the restriction sites BamHI and EcoRI to generate pYES2-NRT1.1B vector. The resulting vector and the empty vector were introduced into yeast strain 135-A-8 (MATα ptr2-3his3A1 leu2A2O lys2A0 uro3A0) and transformed with genome DNA. The relative expression was quantitatively analysed using qRT-PCR to guarantee higher expression of NRT1.1B in NRT1.1B-transgenic strain compared to the empty vector transgenic strain. Transformed yeast cells carrying pYES2-NRT1.1B or pYES2 were incubated in the YPD liquid medium at 30 °C with shaking at 250 r.p.m. until OD₆₀₀ reached 0.6 (about 16–20 h) respectively. The yeast cells were collected by centrifugation at 700 g, and then cultured in YPD liquid medium containing 2 μM SeMet at 30 °C with shaking at 250 r.p.m. for 3 h. The yeast cells were collected by centrifugation and rinsed with sterile H₂O five times to remove the rest SeMet. Finally, the rinsed yeast cells were weighed and digested for Se concentration analysis (Hu et al., 2015).

**SeMet uptake assay in Xenopus laevis oocytes**

The coding region of NRT1.1B (japonica) was amplified and cloned into the Xenopus laevis oocyte expression vector pcSZ2-2 between the restriction sites BamHI and EcoRI and then linearized with Apal. Capped mRNA was synthesized in vitro using the mMESSAGE mMACHINE kit (Ambion, AM1340) according to the manufacturer’s protocol. X. laevis oocytes at stage V–VI were
injected with 46 ng of NRT1.1B cRNA in 46 nL nuclease-free water. After injection, the oocytes were cultured in ND96 medium containing 200 μM SeMet for 6 h and used for SeMet uptake assays (Hu et al., 2015).

**Collection and SeMet determination of xylem sap**

Rice seedlings of different genotypes were cultured in Kimura B nutrient solution for 3 weeks as described above. The seedlings were then placed in absorption solution containing 5 mM MES, 0.5 mM Ca(NO₃)₂ and 50 μM SeMet for 6 h, and then transferred back to nutrient solutions without Se for 1 day. The rice seedlings of different genotypes were decapitated at the base of the shoots with a sharp blade. To avoid contamination with cellular constituents from the cut, the first drops of xylem sap exuded from the cut surface of the decapitated shoots were discarded. The xylem sap was collected manually with micropipettes for 1.5 h after decapitation and temporarily stored at −20 °C. The sap samples were enzymatically hydrolysed as described above, and the SeMet concentration of the sap was analysed by HPLC-ICP-MS (Sun et al., 2010).

**Assay of SeMet transport**

The rice seedlings of different genotypes were placed in absorption solutions containing 5 mM MES, 0.5 mM Ca(NO₃)₂ and 6 μM SeMet for 3 h, and then transferred back to nutrient solutions without Se for 3 days. SeMet concentration was determined in the roots, leaf sheaths and leaf blades, respectively, by HPLC-ICP-MS (Sun et al., 2010), and then SeMet content ratios of leaf blades to roots, leaf sheaths to roots and shoots to roots of NRT1.1B-overexpressing lines OE-31/OE-72 and OEvp-21/OEvp-43 were calculated.

**Assay of Se concentration cultured in selenite solution**

The rice seedlings of different genotypes were cultured in Kimura B nutrient solution containing 2 μM Na₂SeO₃ for 3 days, and then harvested. Se concentration was determined in the roots, leaf sheaths and leaf blades, respectively, by ICP-MS (Zhang et al., 2014).

**Expression assay of OsPT2, OsNIP2.1 and NRT1.1B in NRT1.1B-overexpressing lines**

Rice seedlings of different genotypes were cultured in Kimura B nutrient solution for 2 weeks. The roots were harvested for total RNA extraction to determine the expression of OsPT2 and OsNIP2.1 in the NRT1.1B-overexpressing lines using qRT-PCR. The primers are as following: OsPT2 forward primer, cacaactctccctggtatgc; OsPT2 reverse primer, gaaaccacaatttaccac; OsNIP2.1 forward primer, gggcagcaattcggtagtc; OsNIP2.1 reverse primer, ttctgggaggagctccttc. Similarly, the roots and shoots were harvested to determine the expression of NRT1.1B. Forward primer, ggaggctctcagatctta; reverse primer, agggcgtcttctgttggac (Hu et al., 2015).

**Field experiment**

The rice seedlings of different genotypes were grown in the field in Lingshui County of Hainan Province, China. The spacing between plants was 20 cm. The plot size for each genotype was 10 m², with three replicates. The concentrations of organic matter, available N, P and K in the soils were 22 415, 98.74, 41.42 and 144.05 mg/kg respectively. The concentrations of total Se and available Se were 0.530 mg/kg and 5.78 μg/kg respectively. Available N was determined by diffusion disk method. Soil samples were hydrolysed to NH₄ by 1.2 M NaOH solution and continuously absorbed by 2% H₂O₂, and then titrated by 0.01 M HCl solution. Available P was extracted by an acid mixture of 0.05 M HCl and 0.025 M Na₂HPO₄ and determined by molybdenum antimony colorimetric method. Available K was extracted by 1 M neutral NH₄OAC and determined by flame photometer method. Total Se in soil samples was extracted and determined following Se concentration determination of plant samples as described above. Available Se was extracted by 0.05 M KH₂PO₄ and 0.05 M K₂HPO₄ and determined as described above. The soil type was clay with pH 5.6. The cultural management practices were the same for different genotypes. Upon ripening, rice grains were harvested and dried at 50 °C in an oven, and then the Se concentration was determined by ICP-MS (Zhang et al., 2014). In addition, rice grains of overexpressing lines OEvp-21 and OEvp-43 from a single plant were collected for measurements of grain yield per plant. Randomly picked filled grains were used for 1000-grain weight measurements. Ten and five biological replications were performed for grain yield per plant and 1000-grain weight respectively (Hu et al., 2015).

**Statistical analysis**

One-way analysis of variance (ANOVA) was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) to determine the significant differences (P < 0.05 and P < 0.01) between control and treatments. Statistical differences were assessed by Student’s t-test (Zhang et al., 2014).

**Author contributions**

L. Zhang., C. Chu, Y. Li and H. Ling designed research. B. Hu., K. Deng., X. Gao., G Sun., Z. Zhang., P. Li, W. Wang., H. Li., Z. Zhang., L. Li., Z. Fu., J. Yang., S. Gao. and F. Yu performed research; L. Zhang., K. Deng., X. Gao and B. Hu analysed the data; and L. Zhang wrote the paper.

**Conflict of interest**

The authors declare no competing financial interests.

**References**

Abrams, M.M., Shennan, C., Zasoski, R.J. and Burau, R.G. (1990) Selenomethionine uptake by wheat seedlings. Agron. J. 82, 1127–1130.

Ai, P.H., Sun, S.B., Zhao, J.N., Fan, X.R., Xin, W.J., Guo, Q., Yu, L. et al. (2009) Two rice phosphate transporters, OsPht1;2 and OsPht1;6, have different functions and kinetic properties in uptake and translocation. Plant J. 57, 798–809.

Anderson, J.W. (1993) Selenium interactions in sulfur metabolism. In Sulfur Nutrition and Assimilation in Higher Plants: Regulatory Agricultural and Environmental Aspects (De Kok, L.J., Stulen, I., Rennenberg, H., Brunold, C. and Raurer, W.E., eds), pp. 49–60. The Hague, the Netherlands: SPB Academic.

Anderson, J.W. and Scarf, A.R. (1983) Selenium and plant metabolism. In Metals and Micronutrients: Uptake and Utilization by Plants (Robb, D.A. and Pierpoint, W.S., eds), pp. 241–275. London, UK: Academic Press Inc. Ltd.
Aslam, M., Travi, R.L. and Rains, D.W. (2001) Differential effect of amino acids on nitrate uptake and reduction systems in barley roots. Plant Sci. 160, 219–228.

Botha, C.E., Aoki, N., Scofield, G.N., Liu, L., Furbank, R.T. and White, R.G. (2008) A xylem sap retrieval pathway in rice leaf blades: evidence of a role for endocytosis? J. Exp. Bot. 59, 2945–2954.

Broadley, M.R., Alcock, J., Alford, J., Cartwright, P., Foot, I., Fairweather-Tait, S.J., Hart, D.J. et al. (2010) Selenium biofortification of high-yielding winter wheat (Triticum aestivum L.) by liquid or granular Se fertilisation. Plant Soil, 332, 5–18.

Chantratita, W., Supeepsaarsarnmao, W., Chandeying, V., Kulpstrand, S., Iriangkura, N., Ayudhthaya, B., Ruppajo, S. et al. (2004) Delayed progression to AIDS in volunteers treated with long-term HIV-1 immunogen (Remune) therapy in Thailand. HIV Med. 5, 317–325.

Chen, S., Qu, N., Cao, S.Y., Bauwe, H., Chen, S.Y., Tian, W.Z. and Chu, C.C. (2009) Elrashidi, M.A., Adriano, D.C., Workman, S.M. and Lindsay, W.L. (1987) Selenium in Finnish foods after beginning the use of selenate-supplemented fertilisers. J. Food Comp. Res. 15, 488–499.

Dluzniewska, P., Gessler, A., Kopriva, S., Strnad, M., Novak, O., Dietrich, H. and Combs, G.F. (2001) Selenium in global food systems. Hartikainen, H. (2005) Biogeochemistry of selenium and its impact on food chain quality and human health. Br. J. Nutr. 100, 455–468.

Lee, Y.H., Foster, J., Chen, J., Voll, L.M., Weber, A.P. and Tegeder, M. (2007) AAP1 transports uncharged amino acids into roots of Arabidopsis. Plant J. 50, 305–319.

Leran, S., Varala, K., Boyer, J.C., Churazzi, M., Crawford, N., Daniel-Vedele, F., David, L. et al. (2014) A unified nomenclature of NITRATE TRANSPORTER 1/ PEPTIDE TRANSPORTER family members in plants. Trends Plant Sci. 19, 5–9.

Lu, H.F., McGrath, S.P. and Zhao, F.J. (2008) Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. New Phytol. 178, 92–102.

Lu, H.F., Lombi, E., Stroud, J.L., McGrath, S.P. and Zhao, F.J. (2010) Selenium speciation in soil and rice: influence of water management and se fertilizer form. J. Agric. Food Chem. 58, 11837–11843.

Mckenzie, R.C., Rafferty, T.S. and Beckett, G.J. (1998) Selenium: an essential element for immune function. Immunol. Today, 19, 342–345.

Miller, A.J., Fan, X., Shen, Q. and Smith, S.J. (2008) Amino acids and nitrate as signals for the regulation of nitrogen acquisition. J. Exp. Bot. 59, 111–119.

Rennenberg, H. (2006) Exogenous supply of glutamine and active cytokinin for cellular amino acid uptake in both root epidermis and leaf mesophyll. Plant J. 46, 539–548.

Rentsch, D. (2002) Low and high affinity amino acid H+-cotransporters for cellular import of neutral and charged amino acids. Plant Cell Environ. 25, 1657–1664.

Rayman, M.P. (2004) Use of high-seelenium yeast to raise selenium status: how does it measure up? Br. J. Nutr. 92, 557–573.

Rayman, M.P. (2012) Selenium and human health. Lancet, 379, 1256–1268.

Rostock, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G. and Hoekstra, W.G. (1973) Selenium: biological role as a component of glutathione peroxidase. Science. 179, 588–590.

Ryan-Harshman, M. and Aldoori, W. (2005) The relevance of selenium to immunity, cancer, and infectious/inflammatory diseases. Can. J. Diet Pract. Res. 66, 98–102.

Schwarz, K. and Piltz, C.M. (1958) Factor 3 activity of selenium compounds. J. Biol. Chem. 233, 245–251.

Shin, S.H., Yoon, M.J., Kim, M., Kim, J.I., Lee, S.J., Lee, Y.S. and Bae, S. (2007) Enhanced lung cancer cell killing by the combination of selenium and ionizing radiation. Oncol. Rep. 17, 209–216.

Shinembrenner, H. and Sies, H. (2009) Protection against reactive oxygen species by selenoproteins. Biochim. Biophys. Acta 1790, 1478–1485.
