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Plasma-Membrane-Localized Transporter NREET1 is Responsible for Rare Earth Element Uptake in Hyperaccumulator *Dicranopteris linearis*

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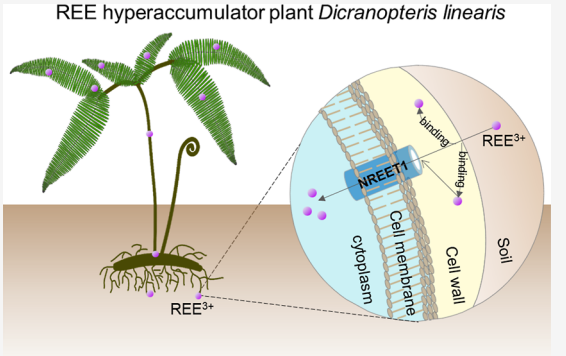
ABSTRACT: Rare earth elements (REEs) are critical for numerous modern technologies, and demand is increasing globally; however, production steps are resource-intensive and environmentally damaging. Some plant species are able to hyperaccumulate REEs, and understanding the biology behind this phenomenon could play a pivotal role in developing more environmentally friendly REE recovery technologies. Here, we identified a REE transporter NRAMP REE Transporter 1 (NREET1) from the REE hyperaccumulator fern *Dicranopteris linearis*. Although NREET1 belongs to the natural resistance-associated macrophage protein (NRAMP) family, it shares a low similarity with other NRAMP members. When expressed in yeast, NREET1 exhibited REE transport capacity, but it could not transport divalent metals, such as zinc, nickel, manganese, or iron. NREET1 is mainly expressed in *D. linearis* roots and predominantly localized in the plasma membrane. Expression studies in *Arabidopsis thaliana* revealed that NREET1 functions as a transporter mediating REE uptake and transfer from root cell walls into the cytoplasm. Moreover, NREET1 has a higher affinity for transporting light REEs compared to heavy REEs, which is consistent to the preferential enrichment of light REEs in field-grown *D. linearis*. We therefore conclude that NREET1 may play an important role in the uptake and consequently hyperaccumulation of REEs in *D. linearis*. These findings lay the foundation for the use of synthetic biology techniques to design and produce sustainable, plant-based REE recovery systems.



KEYWORDS: *rare earth element recovery, plant-based technology, hyperaccumulator, natural resistance-associated macrophage protein*

INTRODUCTION

Despite their name, rare earth elements (REEs) are actually plentiful in the earth's crust and worldwide soils, with an abundance comparable with copper, lead, and zinc.[1] REEs comprise the lanthanides plus scandium and yttrium and can be further subdivided into light REEs (LREEs) defined as series lanthanum (La) to europium (Eu) and heavy REEs (HREEs) spanning gadolinium (Gd) to ytterbium (Yb). Their conductive, optical, magnetic, and luminescent properties make REEs essential for an increasing and diverse array of technological applications, including permanent magnets in wind turbines, catalysts in the petroleum industry and vehicle catalytic converters, electric micromotors, electronic devices, and medical imaging.[2] However, despite their abundance, REEs exist in dilute forms in our environment and require energy intensive and environmentally damaging extraction methods.[3]

Some plant species are able to hyperaccumulate REEs from the surrounding environment and understanding their biology could play a pivotal role in developing more environmentally friendly REE recovery technologies.[4] However, the biology behind how plants take up REEs remains poorly understood. As early as 1917, it was reported that REEs can have a beneficial effect on plant growth,[5] and from the 1970s onward, China has widely applied REEs in agriculture to increase crop productivity, an approach little practiced in other countries.[6] More recently, REEs have been shown to enhance resistance to abiotic and biotic stresses in plants,[7,8] stimulate plant cell growth and biomass production,[9] and there is evidence that REEs have biological activity.[10,11] However, high concentrations of REEs can exert adverse effects on plant growth and

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the use of REEs in agriculture of China has led to an overaccumulation of REEs in these soils.[12] Additionally, mining and processing of REE ores produce huge areas of toxic mine tailings that pose environmental and health risks; coal ash, another polluting waste, can also contain high levels of REEs.[3] These wastes could be remediated, and REEs recovered using plants. But to realize this, it is essential that we understand the biology behind REE uptake by plants, and their subsequent biological functions *in planta*.

Groundbreaking studies have shown that REEs can initiate systemic endocytosis,[13,14] leading to REE nanoparticles directly entering leaf cells.[15] However, even though the roots are the organ directly confronting REEs in the soil environment, how REE ions enter root cells remains largely unknown. The REEs share similar ion radii and chemical properties with calcium (Ca), aluminum (Al), and manganese (Mn), and significant competition for uptake has been reported between REEs and these elements in various plant species.[16−18] Furthermore, most REE hyperaccumulator plant species are also Al or Mn hyperaccumulators.[19] Together these findings suggest that REEs may cross the root cell membranes via Al/Mn transporters, but such hypotheses have not been elucidated at the cellular or molecular levels and are, therefore, far from clear in explaining the regulation of REEs in plants.

The natural resistance-associated macrophage proteins (NRAMP) family members are widespread in different organisms.[20,21] These proteins can transport a wide range of divalent metal substrates, and among many of them play a vital role in the Mn homeostasis in plants, such as *At*Nramp1−*At*Nramp4 in *Arabidopsis thaliana*[22−24] and *Os*Nramp3 in rice.[25] It is noteworthy that, some NRAMP members are found to specifically transport trivalent Al, including *OsNrat1* in rice and *SbNrat1* in sorghum,[26,27] which play critical roles in Al uptake and detoxification in plants. However, whether NRAMP members have REE transport capacity remains unknown.

The fern, *Dicranopteris linearis* accumulates some of the highest levels of REEs reported, with up to 0.7% dry weight of REEs in its leaves.[28] Our previous studies on *D. linearis* localized these elements in the vascular bundles of roots and stems and in the leaf epidermis,[29,30] positing an active regulation of REE uptake, transport, and sequestration in this plant. Moreover, *D. linearis* is also an Al hyperaccumulator,[31] leading us to suspect that NRAMP members could play a role in REE uptake and accumulation in this fern. However, propagation and hydroponic cultivation are time-consuming and technically challenging, and as a result, research on REE hyperaccumulation in this fern has remained elusive. Here, we report the successful hydroponic cultivation of *D. linearis* and analyzed the potential REE transport capacity of four *D. linearis* NRAMP members. Among them, *OP331328* encodes a plasma-membrane-localized transporter which exhibited the strongest REE transport capacity in yeast. This gene is mainly expressed in the roots, where it transfers REEs from the root cell wall to the cytoplasm. We therefore named this gene NRAMP REE Transporter 1 (NREET1). To our knowledge, this is the first REE transporter reported in plants, laying a foundation for enhancing REE phytoextraction through genetically engineered plants.

MATERIALS AND METHODS

Germination, Growth, and Hydroponic Culture of *D. linearis*. Spores of *D. linearis* were collected from REE-enriched soils at ion-adsorption-type REE mines near Ganzhou City, Jiangxi province, southern China. They were surface sterilized with 70% alcohol for 45 s, washed with deionized water five times, then incubated on moist soil at a ratio of ∼0.6 g/m^2. After 2 months, the spores developed into gametophytes. Milli-Q water of which pH was adjusted to 4.2 ± 0.1 with HCl−KOH, was sprayed onto the soil surface daily. After another 5 months, the gametophytes were grown into sporophytes of ∼3 cm in size and transferred into 0.2 strength Hoagland nutrient solution (0.2× HNS, pH = 4.2) with 1 μM KH_2PO_4 instead of 100 μM KH_2PO because *D. linearis* naturally grows on acidic soils with very low P status and has a high P utilization efficiency.[32,33] The growth conditions of spore germination and hydroponic cultivation were as follows: 25/20 °C day/night temperatures, 70% relative humidity, 14 h photoperiod, and 150 μmol s^{-1} m^{-2} light intensity.

One-year-old plants were selected for further hydroponics experiments. For the concentration-dosing experiment, five concentrations of Nd (0, 1, 10, 100, 500 μM Nd) were supplied to the plants as chloride salt in the 0.2× HNS solution (1 μM KH_2PO_4) for 4 weeks. For the low temperature and metabolic inhibitor experiment, the plants were exposed to 100 μM $NdCl_3$ for 3 days under the treatment of the control (25 °C), low temperature (4 °C), or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 100 μM, 25 °C) respectively. CCCP is a widely used metabolic inhibitor, which can completely block fructose protection against cyanide and thereby cause mitochondrial depolarization and rapid ATP depletion.[34] For the ion competitive experiments, the plants were divided into two groups treated with 1 μM Nd or 100 μM Nd. Each group of plants was also treated as control or with 5 mM $CaCl_2$, 100 μM $AlCl_3$, or 100 μM $MnCl_2$ for 2 weeks.[17,35] Verapamil (100 μM), a typical Ca^{2+} channel inhibitor which can potently antagonize the extracellular Ca^{2+} movement through voltage-dependent channels,[36] was also supplied to plants under 1 μM Nd or 100 μM Nd treatment for 3 days. All treatment solution was renewed every 3 days and the pH was constantly kept at pH = 4.2 ± 0.1 to ensure Nd is soluble and is mostly presented in the ionic form (Table S1).[37] After treatments, all plants were harvested individually.

Cloning and Analysis of NRAMP Proteins. To obtain the full-length transcriptome of *D. linearis*, total RNA was extracted from a mixed sample (roots and leaves) of fresh ferns and reversely transcribed into cDNA using the methods described in our previous study.[38] Then, the cDNA was purified by AMPure PB bead and used for SMRTbell library construction. After DNA damage repair and end repair, the cDNAs were ligated to adapters. Once the SMRTbell library was annealed to a sequencing primer, it was bound to polymerase and sequenced on the PacBio Sequel II platform by Gene Denovo Biotechnology Co. Ltd. (Guangzhou, China). Based on the full-length transcriptome, the homologous genes of *OsNrat1*, including *DlNREET1* (OP331328), *DlNramp2*, *DlNramp3*, and *DlNramp4*, were matched and coding sequences (CDS) of these genes were cloned. Amino acid sequence alignments of *D. linearis* NRAMP proteins and *OsNrat1* were carried out in DNAMAN 7.0 software (Lynnon Biosoft, Quebec, Canada). The amino acid sequences of NRAMP proteins from *D. linearis*, *A. thaliana* (*Arabidopsis*), rice (*Oryza sativa* L.), and sorghum (*Sorghum bicolor*) were aligned to construct a phylogenetic tree based on the neighbor-joining methods in MEGA version 11.0.[39] Using T-coffee server,[40] multisequence alignments of different NRAMP

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

proteins were performed and then formatted in ESPript version 3.0.[41] The secondary structure of *Deinococcus radiodurans Dra*Nramp (Q9RTP8) served as a control.[42]

Gene Expression Analysis in *D. linearis*. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was undertaken on plants from two experiments. For the tissue expression analysis, *D. linearis* plants were collected from the field then divided into roots, stolon, stems, and leaves. For the metal-induced expression analysis, seedlings of *D. linearis* were transferred to 0.2× HNS (CK) or 0.2× HNS containing 100 μM $NdCl_3$, $LaCl_3$, $YbCl_3$, or $AlCl_3$ for 7 days, respectively. Each treatment had four replicates. The plants were divided into roots and shoots, washed with deionized water, then ground using a mortar and pestle under liquid nitrogen. A plant total RNA kit (Sigma-Aldrich, USA) was used for total RNA extraction and a HiScript II One Step RT-PCR kit (Vazyme Biotech, Nanjing, China) was used for reverse transcription. Based on the StepOne Real-time PCR system (Applied Biosystems), qRT-PCR analysis was performed using SYBR Green PCR Master Mix (Vazyme Biotech, Nanjing, China). The *D. linearis actin* and *histone* genes were used as internal controls. The primers for qRT-PCR analysis are shown in Table S2.

Yeast Expression. Adapters were added to the CDS of the candidate genes (primers in Table S3), and the PCR products cloned into pYES2 (GAL1 promoter cassette) by recombination, using a Trelief SoSoo Cloning Kit (Tsing Ke, Guangzhou, China). The resulting plasmids or the empty vector pYES2 were introduced into yeast, strain BY4741 (*MATa his2Δ0 met15Δ0 ura3Δ0*). Transformants were selected on synthetic dropout (SD) without amino acids of Ura (U) medium, with 2% glucose as the only C source.

For the growth assay, all yeast cells were first pre-incubated in liquid SD-U medium to about 1.0 OD_{600}. They were then centrifuged at 3000 rpm for 5 min, washed with sterile water for three times and adjusted to the same OD_{600}. Then, 3 μL of 2-fold serial gradient dilutions was spotted on SD-U agar plates containing 1 μM phosphorous (P) and 10 μM REE (Nd, La or Yb). The low P concentration was arranged to maintain the growth of yeast as well as to avoid precipitation with REEs. Yeast cell growth on plates was monitored after incubation at 30 °C for 10 days.

For the REE uptake assay, yeast transformants were cultured in 30 mL of liquid SD-U medium to an OD_{600} ~1.0, harvested, and resuspended in sterile water. The pre-cultured yeast was then added to SD-U without phosphorus and containing 10 μM $NdCl_3$, 10 μM $LaCl_3$, or10 μM $YbCl_3$, then incubated at 200 rpm and 30 °C for 1 h. For the measurement of Al, Zn, Ni, Mn, and Fe(II), their chloride salts were added into the solution with the concentration of 50 μM for each cations and the pH was adjusted to pH = 4.2 for Al and pH = 5.5 for the other metals.[26]

***Arabidopsis* Expressing *NREET1*.** The *DlNREET1* CDS was cloned, using homologous recombination, into the plant expression vector pSN1305 (pCAMBIA1305) under the control of the CaMV 35s promoter. This *P35S-DlNREET1* construct was introduced into *Agrobacterium* strain GV3101 and used to transform *Arabidopsis* via the floral dip transformation method. In brief, the developing floral tissues of *Arabidopsis* were dipped into a solution containing transgenic GV3101, 5% sucrose, and 500 microliters per liter of surfactant Silwet L-77 for around 45 s, once a week, three times in total.[43] The transgenic seeds (T_1 generation) were selected using hygromycin as the selectable marker. Gene transcript levels were quantified using qRT-PCR with *AtActin* (*AT3G18780*) as an internal control.

To investigate the REE accumulation ability of NREET-expressing *Arabidopsis*, seeds were germinated on 1/2 MS medium containing 30 mg/L hygromycin; WT seeds were sown on 1/2 MS only medium. Seeds were germinated and seedlings grown in a growth chamber with a photoperiod of 16 h light/8 h dark, a light intensity of 150 μmol s^{-1} m^{-2}, at 25 °C. Five day old seedlings were transferred to 1/2 MS medium and dosed with REEs as required but without P to avoid precipitating REEs[44] and grown for 7 days. The culture conditions in the chamber were 16 h light/8 h dark at 25 °C.

GFP Fusion and Subcellular Localization. The *DlNREET1* CDS was cloned into the pSAT6A-EGFP vector, producing the *P35S-DlNREET1::EGFP* construct under the control of the *35S* promoter (*P35S*). Rice protoplasts and tobacco (*Nicotiana benthamiana*) expression systems were used for the investigations. Rice protoplasts were isolated from fresh sheath tissues with quick plasmolysis treatment (0.6 M mannitol) and enzymatic digestion (1.5% Cellulase RS, 0.75% Macerozyme R-10, 0.6 M mannitol, 10 mM MES at pH = 5.7, 10 mM $CaCl_2$, and 0.1% BSA).[45] The protoplasts were transiently transfected with *P35S-EGFP* and *P35S-DlNREET1::EGFP* plasmids by using the PEG-mediated approach and incubated in the dark at 28 °C for 12 h. The GFP fluorescence intensity was imaged using a laser scanning confocal microscope (Zeiss LSM 800). Staining dye FM4-64 was used as a plasma membrane marker. The *P35S-DlNREET1::EGFP* plasmids were transiently expressed in the leaves of 3 week old tobacco plants using *Agrobacterium*-mediated infiltration with strain GV3101.[46] Two days after infiltration, fluorescence signals were monitored using a laser scanning confocal microscope.

Separation of *Arabidopsis* Tissues for REE Analysis. The WT and transgenic *Arabidopsis* were germinated on sand moistened with 0.5-strength Hoagland nutrient solution (0.5× HNS) for 14 days and then transplanted to 0.5× HNS for 4 weeks. Plants were divided into two groups and then exposed to 30 μM Nd (pH 5.0) for 24 h. After that, one group was harvested, while the other was transferred into 1/2 HNS with 0 μM Nd for another 24 h before harvesting. Subsequently, shoots were washed with deionized water five times, while cell homogenate of roots was separated into three fractions, including exchangeable apoplastic fraction (F1), symplastic fraction (F2), and nonexchangeable apoplast fraction (F3), following the methods described by Tang et al.[47]

Sample Preparation, Digestion, and Element Determination. For REE-treated plants, roots were desorbed with ice-cold 5 mM $CaCl_2$ solution for 15 min[16] to remove possible residual cations and REEs from the root surface. For REE-dosed yeast, cells were harvested and washed three times with an ice-cold solution of 20 mM MES and 10 mM EDTA (pH = 6), then rinsed three times with ice-cold deionized water.[48] For other metal treatments, yeast cells were washed three times with ice-cold deionized water only. All samples were air-dried and digested according to Liu et al. 2021.[33] Total REE and Al concentrations in yeast or plants were determined using inductively coupled plasma mass spectrometry (NexION350D, PerkinElmer, USA), and the total concentration of Mn, Fe, and Zn in yeast was determined with inductively coupled plasma optical emission spectrometry (Optima5300DV, PerkinElmer, USA).

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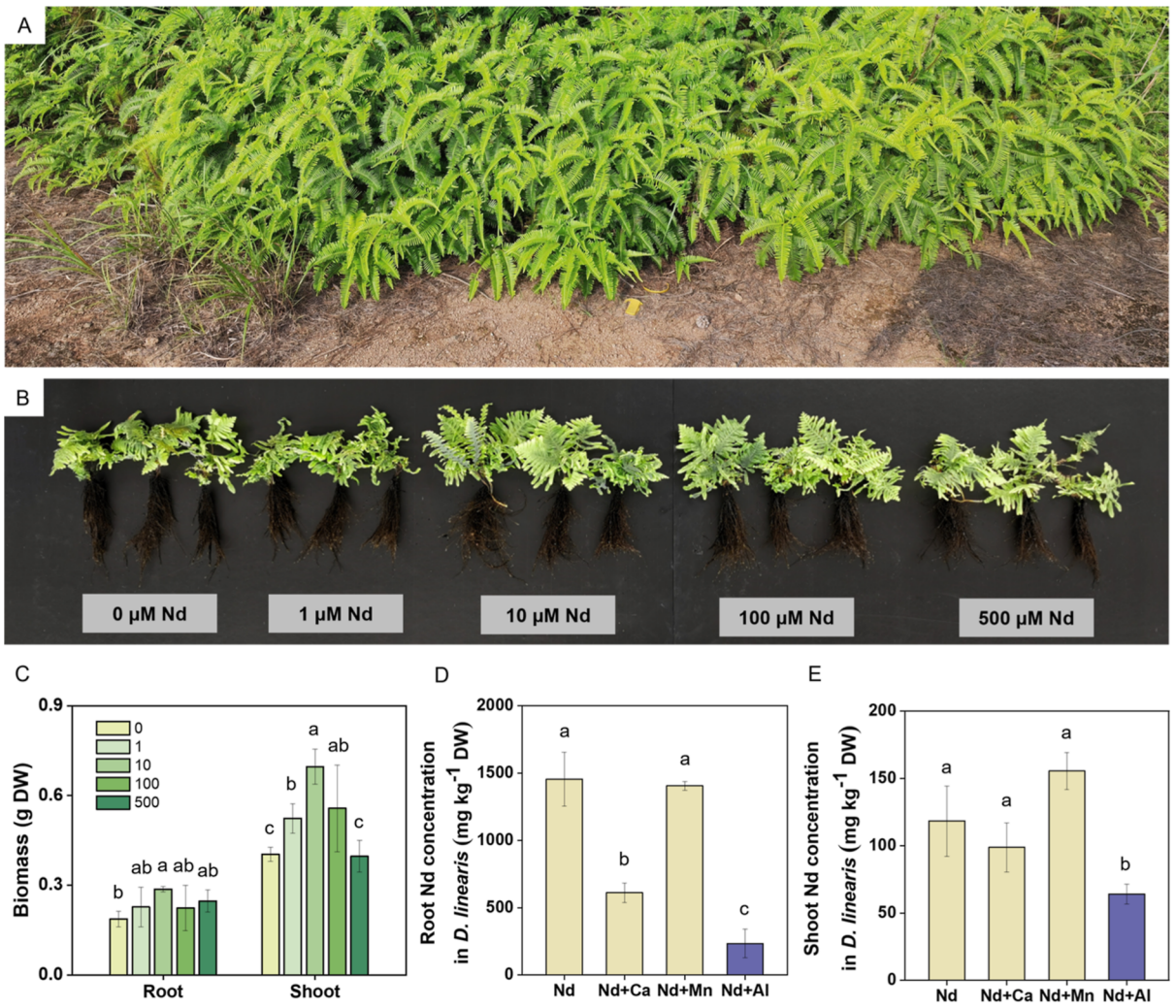

Figure 1. Nd accumulation and tolerance characteristics in hydroponically grown *D. linearis*. (A) Plants growing on ion-adsorption REE mine tailings in southern China. (B) Appearance and (C) biomass of *D. linearis* plants after 4 week exposure to $NdCl_3$. Effect on Nd accumulation in plants dosed with 1 μM $NdCl_3$ alone, or in combination with 5 mM $CaCl_2$, 100 μM $MnCl_2$, or 100 μM $AlCl_3$ in (D) roots and (E) shoots. The values are means ± SDs (n = 3). Different letters on the bars represent significant differences (P < 0.05). DW, dry weight.

RESULTS AND DISCUSSION

REE Uptake in *D. linearis*. Plants were collected from REE mine tailings in southern China (Figure 1A). Our previous studies demonstrated that these plants accumulated more LREEs than HREEs in the shoots,[33] wherein the concentration of LREE neodymium (Nd) ranked the top two among all REEs.[49] Therefore, we chose Nd in our hydroponic experiments. Figure 1B,C shows that the biomass of hydroponically grown *D. linearis* exposed to 500 μM Nd was indistinguishable from that of control plants. However, at the 1, 10, and 100 μM Nd treatments, shoot biomass was significantly higher than the control treatment, with a 73% increase being observed under the 10 μM Nd treatment. Measurement of root and shoot Nd concentrations revealed that levels were similar in roots dosed with 100 and 500 μM Nd, suggesting that capacity had plateaued, whereas shoot Nd concentration continued to increase with Nd increasing from 10 to 500 μM (Figure S1A,B). These results demonstrate that *D. linearis* can accumulate this REE and are in agreement with the previous literature[6] that a low concentration of REE promotes growth.

To investigate the potential pathways related to REE accumulation in *D. linearis*, we first studied the effect of low temperature and inhibitor treatments on Nd uptake by hydroponically grown plants exposed to 100 μM Nd. In plants grown at 4 °C or treated with the CCCP, uptake of Nd was reduced, indicating a biological role of energy-dependent transport systems in plant Nd uptake (Figure S1C). Although some studies have suggested that REE uptake by plants might be linked with Fe uptake,[50] our previous studies on field-grown *D. linearis* have shown that this species accumulates much lower level of Fe in its shoots compared to REE and Al, and no significant correlation between the concentration of REE and Fe was found in *D. linearis* tissues (roots and shoots).[33] Moreover, increasing Nd treatments had no significant impact on the Fe accumulation in *D. linearis* (Figure S2). Hence, we speculated that pathways of Fe might have limited impact on REE accumulation in *D. linearis*. Given that Ca, Al, and Mn uptake routes have been widely implicated in the uptake of REEs,[16−19] we thereby performed competition studies on hydroponically grown *D. linearis* dosed with Nd. In the presence of high (100 μM) Nd, all three metals significantly reduced Nd accumulation in the shoot tissues (Figure S3A,B). Treatment with the Ca channel inhibitor verapamil significantly reduced Nd accumulation at both high and low (1 μM)

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

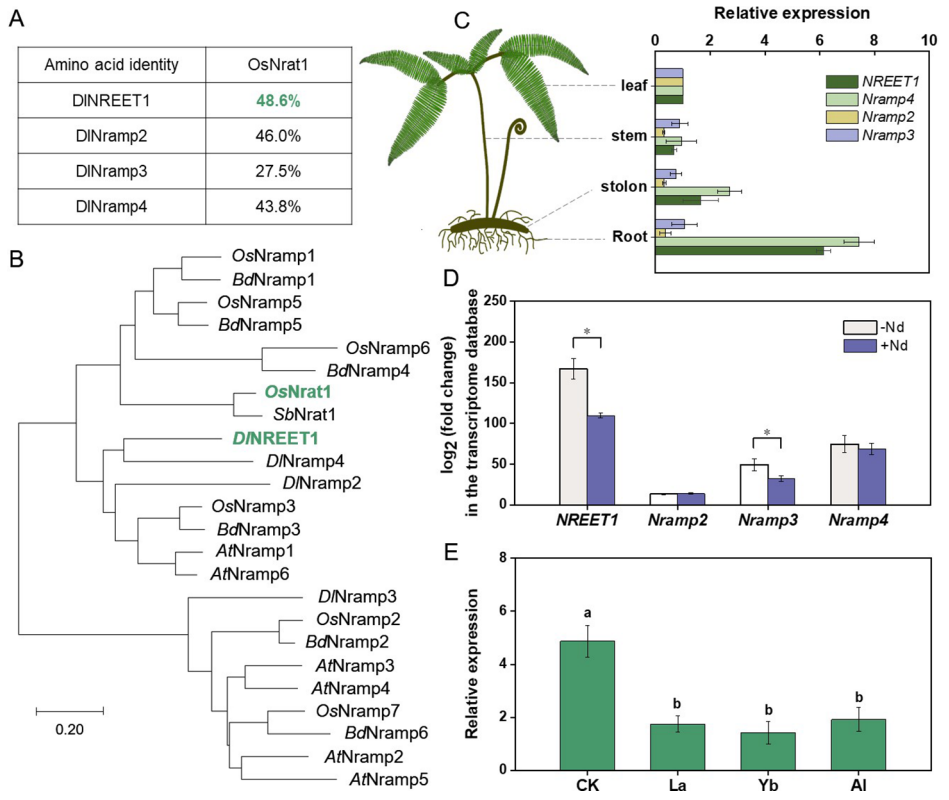

Figure 2. Characterization and expression of *DlNREET1* in *D. linearis*. (A) Comparative amino acid identity between *D. linearis* NRAMP proteins and *OsNrat1*. (B) Phylogenic tree of NRAMP proteins in *D. linearis* (*Dl*), rice (*Os*), *Brachypodium distachyon* (*Bd*), *Sorghum* (*Sb*), and *Arabidopsis* (*At*). (C) Expression of the *NRAMP* genes in field-grown *D. linearis* tissues (relative to the values in the leaves) determined by qRT-PCR. (D) RNA-seq $\log_2$ (fold change) values of *NRAMP*s in the roots of hydroponically grown *D. linearis* with or without exposure to 100 μM Nd for 7 days. (E) Transcriptional patterns of *DlNREET1* in the roots of hydroponically grown *D. linearis* dosed with 0.2× HNS (CK) or 0.2× HNS containing 100 μM $LaCl_3$, $YbCl_3$, or $AlCl_3$ for 7 days, respectively. Values are means ± SD ($n = 4$) and means marked with different letters or asterisks indicate significant differences ($P < 0.05$).

Nd treatment in the roots, but at a low Nd concentration, verapamil did not affect Nd shoot accumulation (Figure S3C,D). At low (1 μM) Nd, treatment with Ca or Al significantly reduced Nd root accumulation; however, only Al treatment significantly reduced Nd shoot concentration (Figure 1D,E). Together, these results indicate a hierarchy of routes for REE uptake in *D. linearis* whereby Al transporters are the predominant mechanism for REE uptake and shoot translocation, with Ca transporters being involved to a lesser degree, and finally Mn transporters.

Identification of Genes Encoding Putative REE Transporters in *D. linearis*. To better understand the molecular mechanisms of REE acquisition and translocation *in planta*, a full-length transcriptome database of *D. linearis* was constructed based on mixed samples (including both roots and shoots) from hydroponically grown and field-grown *D. linearis* plants. Rice (*O. sativa* L.) *Os*Nart1, a NRAMP transporter, plays an important role in Al uptake and tolerance.[26,51] Thus, we used *OsNrat1* as a template to identify four homologous *NRAMP* sequences in *D. linearis*: *DlNREET1*, *DlNramp2*, *DlNramp3*, and *DlNramp4*. A neighbor-joining phylogenetic tree revealed that these four NRAMP members belong to two distinct clades, with *DlNREET1*, *DlNramp2*, and *DlNramp4* being grouped together with *OsNrat1* (Figure 2B). In addition, the protein encoded by *DlNREET1* shared the highest similarity with *Os*Nart1, but the identify was only 48.6% at the amino acid level (Figure 2A).

Expression Analysis of *D. linearis* Candidate REE Transporters. The expression pattern of the four NRAMP members was determined in different tissues using qRT-PCR analysis. The results show that *DlNREET1* and *DlNramp4* were expressed most highly in the roots, with expression levels being relatively lower in the stolons, stems, and leaves. Conversely, *DlNramp2* and *DlNramp3* were expressed at lower levels, with *DlNramp2* being expressed more strongly in the leaves rather than in other tissues, and *DlNramp3* being expressed more constitutively (Figure 2C). The different expression patterns of these genes suggest they have different functions in *D. linearis*. In rice, *OsNrat1* is mainly expressed in the roots transferring Al from the cell wall into the cytosol,[26] *OsNramp2* is expressed predominantly in the leaves, transporting iron (Fe) from the vacuole to the cytosol,[52] while *OsNramp3* is expressed in the nodal vasculature that connects the leaves, stems, and panicles, where it mediates transfer and distribution of Mn.[25]

To establish whether expression of the candidate transporter genes responds to REE exposure, comparative transcriptome and qRT-PCR analysis were performed on hydroponically grown *D. linearis*. The results show that *DlNREET1* and *DlNramp3* in the roots were 1.5-fold and 1.4-fold down-regulated under high (100 μM) Nd treatment, while there were no significant changes in *DlNramp2* and *DlNramp4* expression (Figure 2D). Similarly, *DlNREET1* expression in the roots was significantly lower when treated with the LREE La, the HREE Yb, or Al (Figure 2E). Together, these findings

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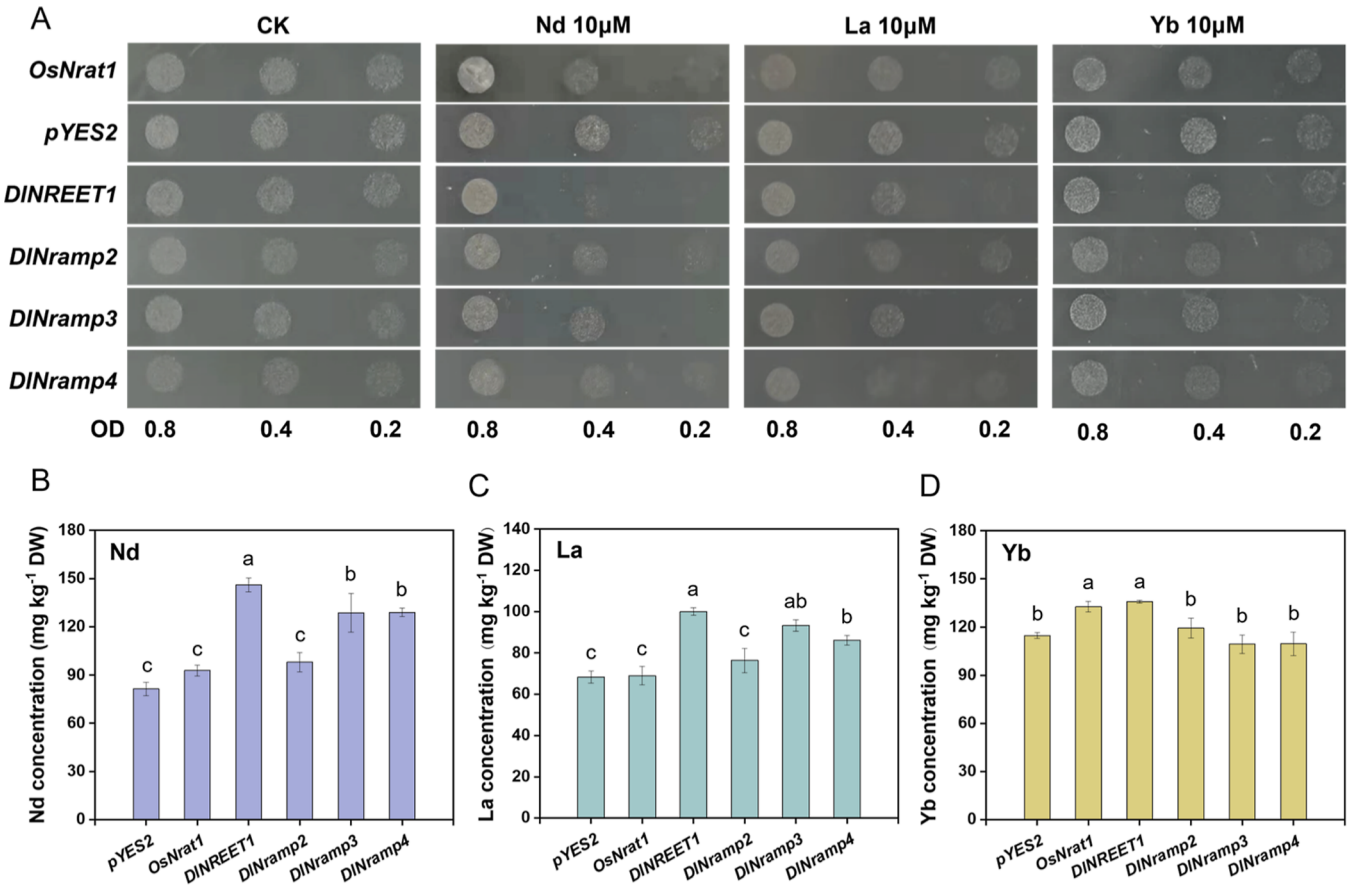

Figure 3. Transport activity of *DlNREET1* for REEs in yeast. (A) Yeast cells (strain BY4741) carrying empty vector pYES2, *OsNrat1*, *DlNREET1*, *DlNramp2*, *DlNramp3*, or *DlNramp4* were spotted onto SD-U agar plates containing 1 μM P, with or without 10 μM $NdCl_3$, $LaCl_3$, or $YbCl_3$. (B) Nd, (C) La, and (D) Yb concentrations in yeast cells after incubation for 1 h in 10 μM solutions containing these metals. Values are means ± SD of three biological replicates. Different letters indicate significant differences ($P < 0.05$). DW, dry weight.

indicate a role for *DlNREET1* in root REE and Al uptake in *D. linearis*. Our previous studies revealed that the hyperaccumulation of silicon (Si) in *D. linearis* leaves plays a key role in REE and Al detoxification.[30,31] However, during the hydroponic experiments, we did not add Si to the solution, which may limit the REE tolerance ability of *D. linearis*. Hence, the downregulation of gene expression induced by REEs and Al could be a mechanism to cope with high metal stress by preventing the sudden accumulation *in planta*.

***D. linearis NRAMP* Expression in Yeast.** To examine whether our candidate *D. linearis* NRAMP transporters had REE transport activity, the CDSs were expressed in yeast (*Saccharomyces cerevisiae*). Empty vector pYES2 and the trivalent Al transporter gene *OsNrat1* were used as controls. As shown in Figure 3A, all yeast transformants exhibited similar growth in the absence of REEs. However, when treated with Nd or La (as representatives of LREE), the growth of *DlNREET1*-, *DlNramp3*-, and *DlNramp4*-transformants was inhibited compared to yeast transformed with the pYES2 empty vector. Conversely, treatment with Yb (as a representative of a HREE) had no significant impact on the growth of the yeast transformants. In comparison, none of the treatments significantly influenced the growth of yeast transformed with *OsNrat1*. The data indicate that in comparison to pYES2, the expression of *DlNREET1*, *DlNramp3*, or *DlNramp4* increased the sensitivity of yeast to REE treatment due to increased accumulation of these elements. To test this hypothesis, the REE concentrations were determined in these yeast transformants. Yeast expressing *DlNREET1*, *DlNramp3*, or *DlNramp4* accumulated significantly more Nd and La than the pYES2 control (Figure 3B,C), while only the *DlNREET1* transformant and *OsNrat1* accumulated more Yb (Figure 3D). These findings further demonstrated that *D. linearis* NRAMP proteins (except *Dl*NRAMP2) has REE transport capacity and favors LREEs over HREEs. Among them, *Dl*NREET1 showed the highest REE transport activity, which increased the yeast Nd, La, and Yb concentration by 80, 46, and 18%, respectively, relative to the pYES2-transformed cells. Considering that *NREET1* is highly expressed in the roots of *D. linearis*, we therefore speculated that *NREET1* might play an important role in root REE uptake.

We also tested the ability of the yeast expressing *D. linearis* NRAMP transporters to take up Al. The results showed that only expression of *DlNREET1* and *OsNrat1* in yeast conferred significantly enhanced uptake of Al compared to the pYES2 control, indicating that NREET1 has a similar Al transport capacity to *OsNrat1* (Figure S4A). Interestingly, expressing *DlNREET1* had no significant effect on accumulation of Zn, Ni, Mn, or Fe in yeast (Figure S4B), indicating that this protein, in common with *OsNrat1*, is unable to transport divalent metals. Previous studies have reported that *OsNrat1* contains a unique metal-binding site (motif B, "Ala−Ile−Ile−Thr"), which is a determinant for the selective transport of

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

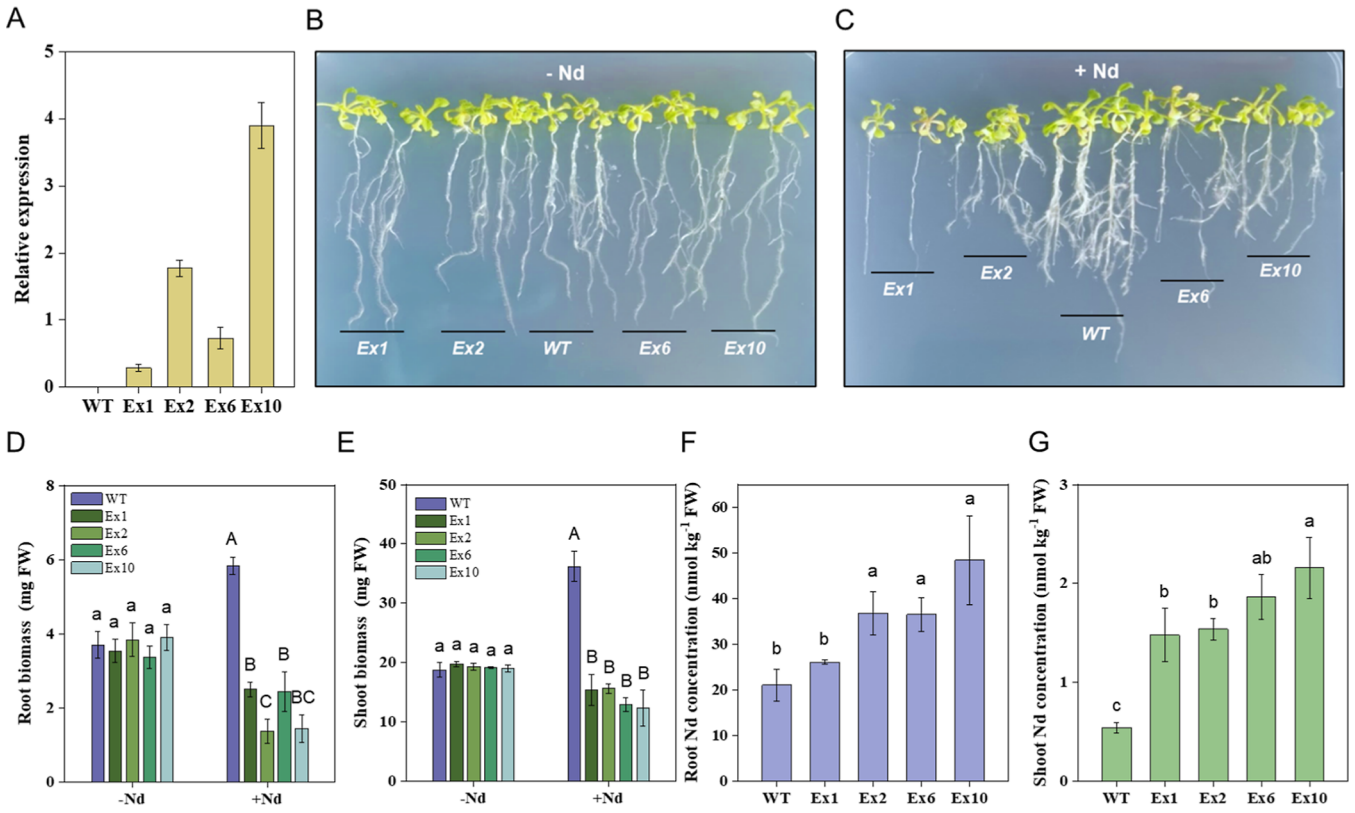

Figure 4. Growth and Nd accumulation of *Arabidopsis* plants treated with or without 1 μM $NdCl_3$ for 7 days. (A) Relative expression of *DlNREET1* in DlNREET1-expressing lines (Ex1, Ex2, Ex6, Ex10) and WT plants by qRT-PCR. (B,C) Appearance and (D,E) biomass of *DlNREET1* transgenic lines and WT plants. (F) Shoot and (G) root Nd concentrations in *DlNREET1* lines and WT plants. Error bars represent SD (n = 3). Different letters represent significant differences (P < 0.05). FW, fresh weight.

trivalent Al.[53] However, our results showed that instead of motif B, *Dl*NREET1 contains a distinct motif A' (Gly−Phe−Ile−Asp), which differs only at the Leu present in *OsNrat1* (Gly−Phe−Leu−Asp), yet it is quite different from Ala−Tyl−Ile−Asp or Ala−Tyl−Leu−Asp in other NRAMP divalent metal transporters (Figure S5). These differences suggest that motif A' might be the key to REE transportation by NRAMP proteins, although the underlying mechanisms need further exploration.

***D. linearis NREET1* Expression in *Arabidopsis*.** To find out whether NREET1 confers REE transport capacity in higher plants, we constitutively expressed the gene in *Arabidopsis*. Analysis using qRT-PCR with gene-specific primers showed that *NREET1* was strongly expressed in four independently transformed lines, while no related transcripts were detected in the wild-type (WT) plants (Figure 4A).

When grown on media without Nd, no significant phenotypic or biomass differences were observed between the transgenic lines and WT plants (Figure 4B,D,E). However, when treated with 1 μM Nd, the growth and fresh biomass of WT plants were significantly promoted, while the growth of the transgenic plants was severely stunted (Figure 4C,D, E). Subsequent analysis revealed that the root and shoot Nd concentrations in the transgenic lines were significantly higher than that in the WT plants (Figure 4F,G), and the increased levels of Nd *in planta* was the likely cause of the reduced biomass observed in the presence of Nd. Following these results, we tested the ability of the *DlNREET1* transgenic lines to accumulate La and Yb. All four transgenic lines accumulated significantly more La in both the roots and shoots than in the WT lines (Figure S6A,B), while no significant differences in Yb root and shoot concentrations were detected (Figure S6C,D). In agreement with our yeast expression studies, these results confirm that NREET1 has LREE transport capacity in plants.

Previous studies reported that both LREEs and HREEs can enter plant cells by endocytosis.[15] However, this pathway does not explain the preferential enrichment of either LREEs or HREEs in different plant species.[19] Our studies using yeast and *Arabidopsis* consistently found that NREET1 preferentially transports the LREEs Nd and La but not the HREE Yb (Figures 3,4 and S6). Moreover, *Dl*Nramp3 and *Dl*Nramp4 also show LREE but not HREE transport capacity in yeast. These findings are consistent with the preferential enrichment of LREEs observed in field-grown *D. linearis*,[33] and REE transport systems such as NREET1 are the key to REE accumulation in plants.

Subcellular Localization of NREET1 in Plants. Plant members of the NRAMP family have different subcellular distributions, including plasma membrane, vacuole, and Golgi apparatus.[22,25] Thus, we expressed enhanced green fluorescence protein (EGFP)-fused *DlNREET1* in rice protoplasts and tobacco leaves (*Nicotiana tabacum*) to determine its subcellular localization. In rice protoplasts, green fluorescence of the expressed fusion protein (*DlNREET1::EGFP*) was localized to the plasma membrane (Figure 5B), while the green fluorescence protein alone (expressed from *P35S-EGFP*) was detected throughout the cells (Figure 5A). The transgenic protoplasts were also stained with the red-fluorescing plasma membrane marker FM4-64, and red fluorescence was seen to coincide with the GFP fluorescence of *DlNREET1::EGFP*

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

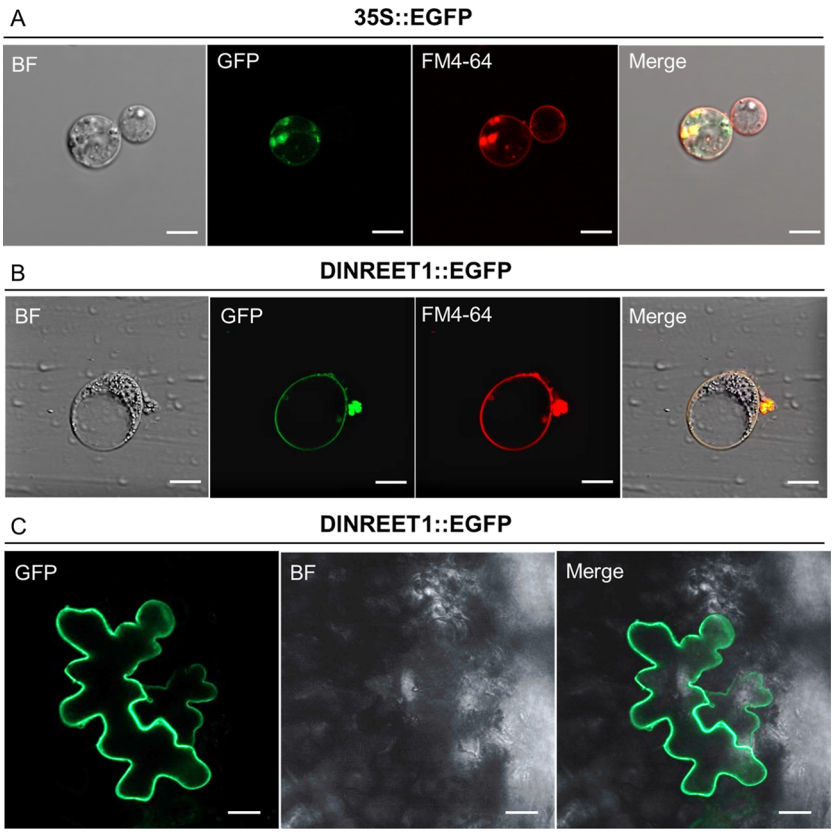

Figure 5. Subcellular localization of (A) *35S::EGFP* and (B) *DlNREET1::EGFP* fusion proteins in rice protoplasts, and (C) *DlNREET1::EGFP* fusion protein in tobacco leaf epidermal cells. BF: bright field, EGFP: enhanced green fluorescent protein, FM4-64: a plasma membrane marker, and merge: overlay of fluorescence.

(Figure 5B). In tobacco leaves, fluorescence from *DlNREET1::EGFP* contoured the cell periphery (Figure 5C). Based on these consistent results from rice protoplasts and tobacco leaves, we propose that *DlNREET1* functions in the plasma membrane of *D. linearis*.

Role of NREET1 in REE Uptake in Plant Roots. To further elucidate the function of NREET1 in plants, we localized the REE distribution within the roots of transgenic *Arabidopsis*. After exposure to 30 μM Nd for 24 h, roots were harvested and separated into three fractions: the exchangeable apoplastic fraction (F1), symplastic fraction (F2), and nonexchangeable apoplast fraction (F3). The Nd concentration in the F2 fraction of the transgenic lines accounted for 19−33% of the root Nd concentration, which was 1.6−2.8 times higher than that of the WT plants. In contrast, in the WT plants nearly 90% of the Nd was found in the F1 and F3 fractions (Figure S7A). Moreover, when these Nd-exposed plants were transferred to nutrient solution without Nd for 24 h, the Nd proportion in the F2 fraction further increased in the transgenic lines, while it had little change in WT plants (Figure S7B). These results support the hypothesis that *NREET1* transports cell-wall-bound Nd into the cells cytoplasm. As *NREET1* is mainly expressed in *D. linearis* roots (Figure 2C) and encodes a cell membrane-localized transporter (Figure 5), we thereby establish that *D. linearis* NREET1 can directly take up REE from the external root environment or transfer cell-wall-bound REE into the cell (Figure S8).

Benefits of the Evolving REE Transport System in Plants. It is interesting to consider why *D. linearis* evolved the ability to hyperaccumulate REEs, specifically why NREET1. In rice, the development of *OsNrat1* functions in Al detoxification, as it can transport excessive wall-bound Al into the root cell for further sequestration.[26,51] In hyperaccumulator plants, it is widely accepted that Al accumulation is a prevailing strategy for plant adaptation to toxic levels of Al solubilized in acidic soils.[54,55] Our results show that NREET1 has the transport capacity for both REEs and Al (Figures 3, S4) and its function in REE uptake is similar to *OsNrat1* for Al uptake in plant roots (Figure S7). As a pioneer species in tropical regions,[31,56] it is not unreasonable to think that *NREET1* might have evolved in response to localized high Al and REE soil conditions. On the other hand, uptake of REEs is known to enhance plant growth, a benefit that could have applied a selective evolutionary pressure in *D. linearis*. It has been reported that in *D. linearis*, REEs can replace magnesium in chlorophyll II, leading to an increased electron transport rate and photosynthetic efficiency.[57,58] Moreover, the accumulation of REEs in the shoots can promote the growth of REE-dependent methanol-catabolizing bacteria.[59] These bacteria have plant-growth-promoting properties, including the ability to release the plant-growth-promoting hormone auxin, fix nitrogen, and solubilize phosphorus.[60,61]

How REE hyperaccumulator plant species are able to take up and withstand the extraordinary levels of these elements have puzzled scientists for a long time. Here, we present the identification and characterization of a REE transporter NREET1. This research lays the foundations on which to further explore REE uptake mechanisms in plants. In addition, humanity's increasing dependence on REEs in technology has raised concerns about their sustainable supply.[2] Phytomining,

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

which uses hyperaccumulator, offers a potential solution to recover valuable REEs from plant biomass.[19] However, hyperaccumulator species generally produce limited amount of biomass and grow in slower rate. Noteworthily, some tolerance plants, such as willow (*Salix* spp.) and *Miscanthus sinensis*, are non-hyperaccumulators but fast-growing species with available genetic transformation protocols. Hence, it has an enormous potential to use synthetic biology tools to create artificial hyperaccumulators. However, to fulfil this, understanding the biology in REE hyperaccumulator plants is the basis. We and others are now sparing no effort to clarify the related molecular mechanism and develop green technologies to recover REEs from plant biomass, with the ultimate aim to harness plant-based systems to supply REEs, and restore mining-damaged and REE-polluted environments.[4]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c09320.

Effect of low temperature (4 °C) or CCCP on Nd accumulation in *D. linearis*; effect on Fe accumulation in *D. linearis* dosed with increasing Nd treatments; effect of metals or inhibitors on Nd accumulation in *D. linearis* under high Nd treatment; metal concentrations (Al, Zn, Ni, Mn, and Fe) in yeast expressing NRAMPs; analysis of the metal-binding sites of 18 plant NRAMP transporters; bacterial model system *DraNRAMP* (Q9RTP8); La and Yb concentrations in *Arabidopsis* expressing *DlNREET1*; distribution of Nd in the root of *DlNREET1*-expressing *Arabidopsis*; schematic representation of NREET1-mediated root REE uptake in *D. linearis*; main Nd speciation in treatment solution; primers for qRT-PCR in this study; and primers used for vector construction in this study (PDF)

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Author Contributions

H.-X.Z. and W.-S.L. contributed equally to this paper. H.-X.Z., Y.T.T., W.-S.L., and Y.C. designed the research. H.-X.Z., D.S., Y.L, Y.L.Y., R.R.L., H.Y.F., and X.C. performed the experiments. H.-X.Z. and W.-S.L. wrote the paper. R.L.Q. provided the funding and resources. All authors discussed the results and commented on the manuscript.

Notes

The authors declare no competing financial interest.

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX