In vivo visualization of nitrate dynamics using a genetically encoded fluorescent biosensor

Yen-Ning Chen¹, Heather N. Cartwright², Cheng-Hsun Ho¹*  

Nitrate (NO₃⁻) uptake and distribution are critical to plant life. Although the upstream regulation of NO₃⁻ uptake and downstream responses to NO₃⁻ in a variety of cells have been well studied, it is still not possible to directly visualize the spatial and temporal distribution of NO₃⁻ with high resolution at the cellular level. Here, we report a nuclear-localized, genetically encoded fluorescent biosensor, which we named NitraMeter3.0, for the quantitative visualization of NO₃⁻ distribution in Arabidopsis thaliana. This biosensor tracked the spatiotemporal distribution of NO₃⁻ along the primary root axis and disruptions by genetic mutation of transport (low NO₃⁻ uptake) and assimilation (high NO₃⁻ accumulation). The developed biosensor effectively monitors NO₃⁻ concentrations at the cellular level in real time and spatiotemporal changes during the plant life cycle.

INTRODUCTION

The plant root is essential to nutrient uptake. Nitrate (NO₃⁻) is a major nitrogen source and is one of the most limiting factors in agricultural production (1, 2). Within the root, NO₃⁻ levels differ markedly between root cell types (3, 4). Under NO₃⁻ limitation, plants can optimize morphological and physiological parameters; for example, root growth can be directed toward nutrient deposits in the soil, the root surface area can be locally increased, or the transporter density on the membrane can be altered. Moreover, metabolic conversion, storage, and translocation of nitrogen compounds are modified (5, 6). To adjust these parameters, plants have to monitor both the external and intracellular NO₃⁻ concentrations to determine NO₃⁻ acquisition needs by plant roots.

NO₃⁻ uptake predominantly occurs from the soil/rhizosphere into roots. Once in a root cell, NO₃⁻ ions can diffuse within the symplasm from cell to cell. NO₃⁻ ions can serve as an osmotic compound or be assimilated in the root to produce organic nitrogen for cellular growth either locally or be loaded into xylem vessels for transport to the shoot (7). NO₃⁻ uptake, the rate of NO₃⁻ acquisition by the plant, depends on the surface area of the root; in addition, the environmental factors that affect root growth will also affect NO₃⁻ capacity. Furthermore, the root system is very plastic, and NO₃⁻ availability itself strongly affects root development. However, we still do not fully understand the most fundamental aspects of NO₃⁻ uptake by plant roots, such as which tissue(s) is(are) responsible for NO₃⁻ uptake, whether NO₃⁻ uptake is distributed all along the root, and whether NO₃⁻ uptake is restricted to specific developmental zones. In addition, the exact intercellular path from the outer root layers toward the central stele has only been hypothesized and not experimentally proven. It has proven difficult to track NO₃⁻ molecules within plant tissue. Some studies have reported NO₃⁻ detection; however, most of these techniques either lack spatial resolution, e.g., radioactive isotope (8, 9) and the Griess method (10), or have limitations to their use, e.g., vibrating electrodes (11, 12), positron-emitting tracer imaging (13, 14), or secondary ion mass spectrometry (15).

Other ions have been monitored in living tissue through Förster resonance energy transfer (FRET)–based biosensors. FRET sensors are fusion proteins that report on a target molecule through interactions with a sensory domain that cause changes in a protein conformation (16). These conformational changes affect the efficiency of energy transfer from a fused FRET donor fluorescent protein to a fused FRET acceptor fluorescent protein. Changes in energy transfer can be detected by measuring changes in the relative intensity of the two fluorescent proteins (ratio change) after excitation of the donor. The ratio change reports target molecule concentration. Here, we report the development of a fluorescent biosensor, NitraMeter3.0, to monitor the dynamics of NO₃⁻ in plants.

RESULTS AND DISCUSSION

NO₃⁻ FRET sensor engineering and optimization

The bacterial NasR protein is a soluble receptor that contains a NO₃⁻ and nitrite (NO₂⁻)–sensing domain (NIT), which serves as a NO₃⁻–binding pocket (17–19). We generated a biosensor by cloning the NIT domain as a Gateway Entry clone and then recombining it with a previously designed Gateway Destination vector (pDR-FLIP39) that carries an enhanced dimerization (ed) variant of Aphrodite (edAFP), as the FRET acceptor, and of enhanced cyan fluorescent protein (edeCFP), as the FRET donor (20). The fusion proteins were expressed in protease-deficient yeast, purified (20), and analyzed in a spectrophotometer for NO₃⁻–dependent alterations in the fluorescence emission curves after FRET donor excitation (Dx) at 428 nm (fig. S1). Within the NIT domain fusion protein, the fluorophores were within Förster distance, as evidenced by resonance energy transfer; however, NO₃⁻ addition did not trigger a significant change in the energy transfer rate between the emission at 530 nm [Dx acceptor emission (DxAm)] and the emission at 488 nm [Dx donor emission (DxDm)] that could act as a FRET ratio change sensor (ΔDxAm/DxDm). The initial emission ratio (ΔDxAm/DxDm) of the NIT domain fusion protein was greater than 1.2 (fig. S1). To further optimize the sensor, we tested the effect of replacing the NIT domain with the entire NasR protein (Fig. 1A). The NasR fusion construct showed a NO₃⁻–triggered increase in emission ratio. The NasR FRET biosensor was named NitraMeter1.0 (NiMet1.0) and reported NO₃⁻ levels with a positive ratio change (ΔDxAm/DxDm) (fig. S2). Fluorescent protein pair variants and different lengths of

¹Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan.  
²Advanced Imaging Center, Howard Hughes Medical Institute Janelia Research Campus, Ashburn, VA 20147, USA.  
*Corresponding author. Email: zcybele3@sinica.edu.tw
Fig. 1. Engineering and specificity for NO$_3^-$ of nitrate biosensor, NiMet3.0. (A) Structural model of NiMet3.0 bound to NO$_3^-$. NasR, a NO$_3^-$-binding protein, was fused via attB1 and attB2 linkers to a fluorescent protein FRET pair (donor, Aphrodite, and acceptor, Cerulean). The NasR protein (purple) representation is from a published structure of NasR [Protein Data Bank (PDB) 4AKK (17)]. The Aphrodite (yellow) representation is from a published structure of Venus [PDB 1MYW (57)] and the Cerulean (blue) representation is from a published structure of Cerulean [PDB 2WSO (58)]. (B and C) Fluorescence emission ratio at 530 nm (B) and emission wavelength scan (C) of purified NiMet3.0 protein with and without NO$_3^-$. The NO$_3^-$ concentration as indicated in the figures. a.u., arbitrary units. (D) Substrate specificity of purified NiMet3.0 treated with the indicated compounds at 5 mM concentrations. Only NO$_3^-$ triggered responses that were significantly different from control (c) (****P < 0.0001, t test). The presented data are means ± SD of six biological repeats. Experiment performed as in (B).
linkers can have marked effects on sensor responses (21–23). In an attempt to optimize NiMet1.0, different FRET pairs including brightness variants and truncation variants and different lengths of linkers fused to either the N or C terminus of the Gateway Destination vectors [pDR-FLIP30, pDR-FLIP39, and pDR-FLIP42-linker (20)] were tested. A FRET pair variant containing citrusine and monomeric Cerulean (mCer) was consistently NO$_3^-$ responsive; we named this biosensor variant NiMet2.0 (fig. S3). NasR with LI2 linkers showed a larger NO$_3^-$-triggered response when fused to the citrusine/mCer pair (fig. S3). Furthermore, NasR with no L12 linkers sandwiched by Aphrodite t9 (AFPt9) and mCer (pDR-FLIP30) yielded the highest ratio change and the lowest FRET initiation ratio; this variant was thus named NiMet3.0 (Fig. 1B). Considering the crystal structure of NasR (17) and our observed DxAm/DxDm values for NiMet3.0 (hereafter referred to as NiMet3.0 emission ratio) with and without NO$_3^-$ (Fig. 1C), we hypothesize that NiMet3.0 switches from a low-FRET to high-FRET average state upon binding to NO$_3^-$.

**Kinetics, pH, selectivity, and nonresponsive NiMet3.0**

To test the specificity of NiMet3.0 to NO$_3^-$, we examined different forms of nitrogen and other anions. Neither other anions nor other nitrogen forms, like ammonium or a peptide, triggered emission ratio changes; thus, the NiMet3.0 sensor is specific to NO$_3^-$ (Fig. 1D). To determine the dynamic range of NO$_3^-$ detection by NiMet3.0, we measured the dissociation constant ($K_d$) of purified NiMet3.0 in vitro by tracking dose-dependent changes in NiMet3.0 emission ratios for NO$_3^-$ (Fig. 2A). The sensitivity of NasR for NO$_3^-$ is in the micromolar to millimolar range (19). The $K_d$ of NiMet3.0 was $\sim$90 $\mu$M for NO$_3^-$ and reached a maximum at NO$_3^-$ concentrations above 1 mM (Fig. 2A). This affinity is comparable with the NasR sensitivity for NO$_3^-$.

Nonresponsive variants of NiMet3.0, an important control of NiMet3.0 specificity, were generated via mutation of NasR residues involved in NO$_3^-$ binding (Fig. 2B). NiMet3.0-R49A, NiMet3.0-R50A, NiMet3.0-R176A, and NiMet3.0-R236A carry alanine substitutions in the predicted NO$_3^-$-binding pocket of NasR based on the crystal structure of the NasR protein and have been shown to disrupt NO$_3^-$ responses (17). NiMet3.0-R49A and NiMet3.0-R236A still showed detectable response to NO$_3^-$ but with lower emission ratios compared with NiMet3.0, whereas NiMet3.0-R50A and NiMet3.0-R176A, the substitution in the NasR-binding pocket, showed no responses to NO$_3^-$, likely due to disrupted salt bridges that function in the interaction with NO$_3^-$ (Fig. 2C) (17). The above mutant biosensors are evidently nonresponsive to NO$_3^-$ and carry a NO$_3^-$ binding pocket that is predicted to be nonresponsive in planta with endogenous NO$_3^-$.

Together, these data strongly support the hypothesis that NiMet3.0 specifically measures NO$_3^-$ concentrations and can report the dynamics of changes in NO$_3^-$ levels.

To test the specificity of the NiMet3.0 response to NO$_3^-$ in planta, we generated stable transgenic *Arabidopsis* lines expressing either NiMet3.0 or the nonresponsive control NiMet3.0-R176A (under
the control of the strong constitutive CaMV35S). The root tips of 6-day-old seedlings from both lines germinated and grown in nitrogen-free medium with exogenous NO$_3^-$ pulses directly to the primary root for 5 min were examined. Transgenic lines expressing NiMet3.0, but not NiMet3.0-R176A, showed significant emission ratio changes to NO$_3^-$ in roots (Fig. 2D; quantification in Fig. 2E), indicating that NiMet3.0 can specifically detect NO$_3^-$ in plants.

For the generation of high-sensitivity FRET sensors, many parameters are critical, such as sensory domain for affinity and specificity, fluorescent proteins for brightness and ligand-induced FRET changes, and linkers for the effect on sensor responses (16, 24, 25). Here, we successfully engineered NiMet1.0 responses to NO$_3^-$ and further optimized the sensor to create NiMet2.0 and NiMet3.0. NiMet3.0 had a bigger emission change ratio, a better signal-to-noise ratio, and a lower initiated ratio by iterative optimization with ligand-binding domains, linkers, and FRET donor and acceptor fluorescent proteins (Fig. 1). Replacing key NO$_3^-$-binding residues in the ligand-binding pocket of NasR with alanine, we generated a nonresponsive sensor of NiMet3.0, which showed no emission ratio changes to NO$_3^-$ pulses (Fig. 2, B and C). Moreover, NiMet3.0 did not respond to a variety of other nitrogen sources and anions, e.g., sulfate, sulfite, selenite, or molybdate, or chloride. Chlorate is structurally similar to NO$_3^-$ as an analog and is an efficient substrate for NO$_3^-$ reductase (Fig. 1D). Thus, the failure of chlorate to trigger NiMet3.0 emission ratio change in vitro was probably caused by the different charge with chlorate being more electronegative than NO$_3^-$ (26) and/or NasR exhibiting considerable selectivity for inducers (17). Corresponding results were shown in the roots of a nonresponsive sensor of a NiMet3.0 transgenic plant; these results support the notion that NiMet3.0 specifically detects NO$_3^-$ in plants (Fig. 2, D and E). The concentration of NO$_3^-$ in plants varies. The cytoplasm is an important compartment for NO$_3^-$ events. When provided with unlimited supplies of NO$_3^-$, NO$_3^-$ concentrations in the root or shoot can reach up to 100 mM. Most of the NO$_3^-$ is stored in the vacuole (27). The $K_d$ of NiMet3.0 was ~90 $\mu$M for NO$_3^-$ (Fig. 2A). In the future, we propose that the parameters outlined above will be suitable for manipulation to engineer sensors with different affinities to NO$_3^-$ detection.

Expression and characterization of nlsNiMet3.0 in planta

It is assumed that NO$_3^-$ and other small molecules/ions readily diffuse between the cytosol and nucleoplasm via nuclear pores; thus, a sensor targeted to the nucleus will allow the analysis of NO$_3^-$ accumulation in the combination of these compartments, which is effectively cytosolic. To assess the control of NO$_3^-$ distribution in planta, we generated stable transgenic Arabidopsis lines expressing a nuclear-targeted variant of NiMet3.0 (nlsNiMet3.0) under the control of a promoter fragment previously shown to direct broad expression [p16 (28)]. Expression of nlsNiMet3.0 did not result in detectable phenotypic changes in seedlings or plants (Fig. S4). Purified nlsNiMet3.0 showed similar in vitro responses to NO$_3^-$ as NiMet3.0, and exposure to NO$_3^-$ pulses under different pH values from 5.5 to 7.5 had no effect on the emission ratio (Figs. S5 and S6A). These data suggest that the nlsNiMet3.0 sensor can be a highly useful tool in studies of plant development and growth. The emission ratio of nlsMiMet3.0 in the apical meristem zone of primary roots, which were grown in nitrogen-free medium for 5 days with NO$_3^-$ addition for 5 min or with NO$_3^-$ addition for 5 min and removal for 15 min, respectively, was examined. When the emission ratios of primary root cells exposed to NO$_3^-$ pulses were recorded, nlsNiMet3.0 showed a rapid response to NO$_3^-$ pulses, and the signal was reversible when NO$_3^-$ was withheld (Fig. 3A). We further examined the responses of nlsNiMet3.0 in roots to addition of various exogenous concentrations of NO$_3^-$ for 5 min as described above. In roots, the $K_d$ of nlsNiMet3.0 was ~130 $\mu$M for NO$_3^-$ (Fig. 3B and fig. S7). This affinity was comparable to the NiMet3.0 affinity for NO$_3^-$ in vitro. It should be noted that the apparent correlation in planta is consistent with signal site binding with saturation, but additional experiments will be needed to define the absolute concentrations. The NasR protein responded equivalently to both NO$_3^-$ and NO$_2^-$ (18). Purified NiMet3.0 in vitro responded to NO$_3^-$ with $K_d$ ~ 2 $\mu$M (fig. S6, A and B); however, no significant emission ratio changes were observed in primary root cells exposed to NO$_2^-$ pulses (fig. S6, C and D). These data support the results above, suggesting that NiMet3.0 responds specifically to NO$_3^-$ pulses (Figs. 2D and 3, A and B).

NO$_3^-$ is a significant source of nitrogen for bacteria and plants. NasR-encoding protein, which contains the NIT domain, controls NO$_3^-$ and NO$_2^-$ assimilation in Klebsiella oxytoca (18). NarX is another NO$_3^-$ and NO$_2^-$-binding protein that controls NO$_3^-$ and NO$_2^-$ respiration in proteobacteria (29). NasR and NarX protein are both highly selective for NO$_3^-$ and NO$_2^-$, and neither responds to chlorate. Moreover, NarX discriminates efficiently between NO$_3^-$ and NO$_2^-$, whereas NasR responds equally well to both. Our green fluorescent protein (GFP)–based NiMet3.0 sensors responded to NO$_3^-$ and NO$_2^-$, whereas NiMet3.0 detected NO$_3^-$ and NO$_2^-$ under various environmental conditions and genetic analyses, such as different NO$_3^-$ concentrations and Atnar2.1 mutant.

Live-cell imaging of nlsNiMet3.0 response to endogenous and exogenous NO$_3^-$ in roots

To explore whether NiMet3.0 is suitable for measuring NO$_3^-$ distribution in plants, we investigated nlsNiMet3.0 emission ratios around the central section (~1.5 $\mu$m) of the apical meristem and the transition zones in the primary root axis of wild-type Col-0, a NO$_3^-$ transporter...
plates without (as mock) or with 10 μM of NO₃⁻ at pH 5.5 and exposed to long days (16-hour light/8-hour dark) for 5 days (Fig. 3C). NO₃⁻ uptake in root cells requires NO₃⁻ transporters (36). In wild-type roots, we observed an overall higher emission ratio in seedlings grown on NO₃⁻-containing agar compared with those grown without NO₃⁻ (nitrogen-free agar plates). There was an apparent gradient of NO₃⁻ in the root tip, with high nlsNiMet3.0 emission ratios in the apical meristem zone that reduced to lower nlsNiMet3.0 emission ratios in the root transition zone (Fig. 3C), although local variation was observed. As expected, the NO₃⁻ transporter npf6.3 mutant plants showed lower nlsNiMet3.0 emission ratios in all root zones with or without NO₃⁻ in the medium compared to the wild type, supporting the idea that NP6.3 functions as a major NO₃⁻ transporter bringing external NO₃⁻ into roots. Furthermore, there was an overall increase of nlsNiMet3.0 emission ratios with NO₃⁻ treatment in the root of the nia1nia2 mutant compared to that in the wild type and higher nlsNiMet3.0 emission ratios in the cortical cells of the transition zone (Fig. 3C), suggesting an area of higher NIA1/NIA2 protein or activity levels in the root. Our findings support the idea that nlsNiMet3.0 is potentially suitable for measuring NO₃⁻ distribution in planta. Quantification results corresponding to Fig. 3C are shown in Fig. 3D.

To explore which tissue(s) or zone(s) along the root is (are) responsible for NO₃⁻ uptake, a central section of the Col-0 primary root axis as described above underwent short-term exogenous NO₃⁻ addition/removal. We analyzed nlsNiMet3.0 emission ratios in roots of Arabidopsis seedlings, which were germinated and grown on agar plates at pH 5.5 without nitrogen for 5 days, before NO₃⁻ pulsing, 5 min after NO₃⁻ pulsing, and 15 or 30 min after treatment with exogenous NO₃⁻ during external washout. Similar to the long-term NO₃⁻ growth results shown in Fig. 3C, overall higher nlsNiMet3.0 emission ratios were rapidly observed in the root meristem zone with exogenous NO₃⁻ applied for 5 min (Fig. 4A), although the differential nlsNiMet3.0 emission ratios across the root may result from the competing processes of influx, efflux, xylem and vascular loading, and NO₃⁻ reduction. Exogenous treatment of Arabidopsis roots with NO₃⁻ did not increase nlsNiMet3.0 emission ratios in the endodermis cells, whereas it triggered increased nlsNiMet3.0 emission ratios in the epidermis, pericycle, and stele cells, with highest ratios seen in the cortex cells (Fig. 4B). After washing out the exogenous NO₃⁻, the increased nlsNiMet3.0 emission ratio was rapidly reduced in all root cells. After washout, the cortex cells in the root meristem maintained relatively high levels of NO₃⁻ (Fig. 4, A and B). It should be noted that, after accumulation of exogenous NO₃⁻, nlsNiMet3.0 was able to report the depletion of NO₃⁻ from all types of cells of the roots (Figs. 3A and 4, A and B).
The accumulation of exogenously applied NO$_3^-$, detected by nlsNiMet3.0 in the nuclei of root cells, reflects an accumulation of NO$_3^-$ in the cytosol/nucleoplasm and a balance of NO$_3^-$ net flux between import and depletion activities, for example, metabolism, export, and compartmentation. To quantify this cooperative activity with high spatiotemporal resolution, we performed time-course experiments on Arabidopsis roots using light-sheet microscopy, a microfluidic device that allows imaging of roots growing in fluorinated ethylene propylene (FEP) tubes with a perfusion control system (fig. S8).

Arabidopsis seedlings were germinated and grown in 1/20 strength Murashige Skoog (MS) medium at pH 5.5 for 5 to 6 days and then perfused with the medium without nitrogen for another 1 to 2 days. After 90 min of perfusion with 10 µM of NO$_3^-$, the nlsNiMet3.0 emission ratio in the meristem zone of the primary root was nearly saturated, indicating a dynamic balance of NO$_3^-$ net flux in the roots or concentrations of NO$_3^-$ in cytosol/nucleoplasm over the capacity of nlsNiMet3.0 (Fig. 4, C and D). With washout, the emission ratio rapidly reduced back to the initial levels (Fig. 4, C and D, and movie S1). These results also indicated that the steady-state concentrations in the cytosol/nucleoplasm were achieved in 90 min of perfusion with 10 µM of NO$_3^-$.

Investigation of nutrient acquisition has relied heavily on techniques that integrate uptake over the entire root system. Unfortunately, this approach fails to reveal which regions of the root are actually involved in the uptake process. The localization of uptake along the root axis correlates with root development, structure, metabolism, and transport processes. It is also reasonable to expect that cellular biochemistry and metabolic requirements may also vary with the position along the root axis. Net fluxes of NO$_3^-$ into the roots vary both with position along the root axis and with time. These variations may not be consistent in different plants, in which different roots show different temporal and spatial patterns of uptake (12) and NO$_3^-$ activity (38). Our new genetically encoded fluorescent sensor, NitraMeter (NiMet), that monitors the net NO$_3^-$ fluxes in real time in the cellular or subcellular compartments with high spatiotemporal resolution in a minimally invasive manner in living cells provides a solution and enables determination of uptake of NO$_3^-$, steady-state net NO$_3^-$ fluxes, and NO$_3^-$ dynamics in the cytosol/nucleoplasm in roots with high spatial and temporal resolution. When exogenous NO$_3^-$ is pulsed to roots, a rapidly increased emission ratio of NiMet3.0 in Col-0 suggests the high accumulation of NO$_3^-$ in Col-0 root (Figs. 2, D and E, 3, A and B, and 4). This is possibly due to the higher rate of NO$_3^-$ uptake activity by transporter as the seedlings were grown under no- or low-nitrogen conditions. The higher emission ratio in the meristematic zone of primary root in Col-0 suggests that the meristematic zone is mainly responsible for uptake of external NO$_3^-$ into the root. Many NO$_3^-$ transporters dominantly expressed in the primary root have been identified and functionally characterized in the past (36). The results of the responses of nlsNiMet3.0 in npf6.3 mutant (Fig. 3, C and D)
also support the function of NPF6.3 as a major NO$_3^-$ transporter in primary root (39) even under different growth conditions. Moreover, the higher and faster emission ratio in root cortex cells compared to all other cells after exposure to NO$_3^-$ (Fig. 4, A and B) suggests a higher rate of NO$_3^-$ uptake and/or transport. The endodermis cells in the root showed a relatively high NO$_3^-$ accumulation compared to all other cells when seedlings were grown under nitrogen-free conditions (0 min; Fig. 4B); meanwhile, the level of NO$_3^-$ accumulation after exposure to exogenous NO$_3^-$ pulses increased slowly. These data suggest that NO$_3^-$ accumulation in the endodermis cells in the primary root may play an important role as a NO$_3^-$ hub for the plant to respond and adapt to various environments accordingly. AtNPF1.3, which is highly expressed in the endodermis cells in the root, has recently been demonstrated to be a NO$_3^-$ transporter in vitro (40). It is also known that NO$_3^-$ is a potent signaling molecule that regulates global gene expression and many physiological processes, such as root architecture and flowering (1). The cross-talk among NO$_3^-$ accumulation and NO$_3^-$ signals in response to nitrogen availability changes in different cell types in the root, especially in the endodermis cells, will need further investigation.

In addition, a higher emission ratio was observed in the transition zone of nia1nia2 root, suggesting that the NO$_3^-$ reductase levels or activity is higher. NO$_3^-$ reductase is the key enzyme responsible for NO$_3^-$ to NO$_2^-$ reduction in plant cells (41). In nia1nia2 mutant, higher emission ratios of nlsNiMet3.0 in roots indicated higher accumulation of NO$_3^-$ (Fig. 3, C and D), supporting the idea that a comparison of uptake rates for wild-type and nia1nia2 roots would provide insight into rates of net flux of NO$_3^-$ (Fig. 4, A and B). The expression of NO$_3^-$ reductase is the key enzyme responsible for NO$_3^-$ assimilatory enzyme activity (45–47). Notwithstanding, the expression of NO$_3^-$ reductases is regulated by various factors, resulting in a diurnally differential expression pattern; thus, a comparison of uptake rates and investigation of whether/how NO$_3^-$ effects nlsNiMet3.0 in wild-type and nia1nia2 mutant roots would provide insight into the rates of uptake and subsequent reduction. Furthermore, whether the remaining activity is attributed to vacuolar sequestration will need further exploration.

To be incorporated into amino acids, once NO$_3^-$ is taken into cells by transporters, NO$_3^-$ is reduced to NO$_2^-$ in the cytosol (pH ~ 7.5) by NO$_3^-$ reductase, and then NO$_2^-$ is reduced to NH$_4^+$ in the plastids or chloroplasts (pH ~7 to 8) by a NO$_2^-$ reductase. NO$_3^-$ can also be transported into vacuoles (pH ~5 to 7) for storage or transported to the shoot by the xylem (pH ~5 to 6) or phloem (pH ~7 to 9). In animals, including humans, NO$_3^-$ and NO$_2^-$ are recognized as being inert oxidants of nitric oxide, which is a key signaling factor in physiology including vascular homeostasis, neurotransmission, and host defense (24, 48). As nlsNiMet3.0 was found to be less sensitive to pH between 5.5 to 7.5 in vitro, nlsNiMet3.0 could be less sensitive to pH between 5.5 to 7.5 in vitro, nlsNiMet3.0 could potentially be applied to a wide variety of living cells and organisms including plants and animals to provide insights into NO$_3^-$ dynamics.

GFP-based sensors enable monitoring of flux into intact cells. However, to generate a detailed flux model, further information will be required such as the nature and kinetics of the contributing transporters as well as the contribution of vacuoles. NO$_3^-$ transporters, which have been intensively studied in the past decades in plants (36), are placed in strategic positions to control how much NO$_3^-$ can enter a given cell at a given point in time; however, it is still difficult to know where the modifications take place and to determine the effect of each step of transportation. Recently, ratiometric fluorescent NO$_3^-$ sensors for activity of NO$_3^-$ transporters named NItrAc1 and NItrAc-NPF1.3 have been reported to be able to track the movement of NO$_3^-$ through the cell membrane (40, 49). Here, we report NitraMeter3.0 sensor (NiMet3.0) as a new and highly useful tool that can be used in living plant roots to quantify NO$_3^-$ concentrations and dynamics (fig. S9). In the future, it is hoped to test Arabidopsis mutant lines for NO$_3^-$ reductase and various transporters expressing NItrAc1, NItrAc-NPF1.3, and NiMet3.0 sensors in the roots.

**MATERIALS AND METHODS**

**DNA constructs**

The construction of the sensor expression vector has been described (50). Constructs were inserted by Gateway LR reactions into the yeast expression vectors pDRLFip30, pDRLFip39, pDRLFip42-linker, and pDRLFip-GW (Gateway). The pDRLFip30 vector sandwiches the insert between an N-terminal AFP9 variant (25), with nine amino acids truncated off the C terminus, and a C-terminal mCer (51). pDRLFip39 sandwiches the inserted polypeptide between an N-terminal enhanced dimer Aphrodite t9 (edAFP9) and C-terminal fluorescent protein enhanced dimer, with seven amino acids and nine amino acids truncated from the N terminus and the C terminus of eCyCyan (17, ed.eCFP9), respectively. pDRLFip42-linker carries an N-terminal citrine and a C-terminal mCer (51). The pDRLFip42 vector was digested with Kpn I (New England Biolabs) for insertion of additional linker sequences (Arg–Ser–Arg–Pro–Thr–Arg–Pro–Gly–Glu–Leu–Gly–Thr) to generate the pDRLFip42-linker vector. The full-length open reading frame of NasR, the NIT domain of NasR, or NasR carrying point mutations from K. oxytoca (17) in the pDONR221 Gateway Entry vector was used as sensory domains for creating the NO$_3^-$ sensors NiMet-NIT, NiMet1.0, NiMet2.0, NiMet3.0, nlsNiMet3.0, or NiMet3.0-NRs. The yeast expression vectors were then created by Gateway LR reactions between different forms of pDONR221-NasR/NIT and different pDRLFip-GWVs, following the manufacturer’s instructions.

**Generation of NiMet3.0-NR mutants**

The NO$_3^-$ binding domain of the NasR Entry clone for NiMet3.0 was altered using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s instructions to generate the NiMet3.0-NR mutations. Primers for site-directed mutagenesis of NiMet3.0 to create NiMet3.0-NR were as follows: R49A, 5′-catatgctgctgtgcacgggagccatgtaa-3′ (forward) and 5′-attactgtgctgcaccgcctgacactgcatagt-3′ (reverse); R50A, 5′-gtactgtgctgctgtgcacgggagccatgtaatctg-3′ (forward) and 5′-cattactgtgctgcaccgcctgacactgcatagtc-3′ (reverse); R176A, 5′-ccgggtctagtctgaggggagccagctttagctg-3′ (forward) and 5′-caggtattactgtgctgcaccgcctgacactgcatagtc-3′ (reverse); R176A, 5′-ccgggtctagtctgaggggagccagctttagctg-3′ (forward) and 5′-caggtattactgtgctgcaccgcctgacactgcatagtc-3′ (reverse); R236A, 5′-gagattgagcagctggcctgtgtc-3′ (reverse) and 5′-gagattgagcagctggcctgtgtc-3′ (reverse).
Expression of sensors in yeast

A Saccharomyces cerevisiae strain BJ5465 [American Type Culture Collection, 208289 (MATa ura3-52 trp1 leu2-Δ1 his3-Δ200 pep4 : HIS3 prb1-Δ1.6 R can1 GAL) (52), obtained from the Yeast Genetic Stock Center (University of California, Berkeley, CA), was transformed with pDRFlips yeast expression plasmids using a lithium acetate transformation protocol (53). Transformed yeast was selected on solid yeast nitrogen base (YNB; minimal yeast medium without nitrogen; Difco) supplemented with 2% glucose and –ura dropout medium (Clontech). Single colonies were grown in 5 ml of liquid YNB supplemented with 2% glucose and –ura dropout under agitation (230 rpm) at 28°C until optical density at 600 nm ~ 0.8 was reached for fluorescence analysis of sensor expression and for metal affinity chromatography purification of sensors. Yeast strains expressing sensors were grown in 30-ml cultures in –ura dropout medium in 50-ml culture tubes.

Fluorescence analysis of purified sensors

Biosamples were purified by metal affinity chromatography. Yeast lysates were diluted 1:2 in 50 mM Mops and 10 mM imidazole (pH 7.4) and then filtered through a 0.45-µm polyethersulfone (PES) filter and bound to Poly-Prep chromatography columns (Bio-Rad) containing His-Pur Cobalt resin (Bio-Rad). Columns were then washed twice with 50 mM Mops and 10 mM imidazole (pH 7.4) and eluted in 50 mM Mops and 150 mM imidazole (pH 7.4). Samples were diluted in 50 mM Mops (pH 7.4). Fluorescence was measured in a fluorescence plate reader (M1000, Tecan, Austria), in bottom-reading mode using a 7.5-nm bandwidth for both excitation and emission (54, 55). Typically, emission spectra were recorded (λ_em, 470 to 570 nm; step size, 5 nm). To quantify fluorescence responses of the sensors to substrate addition, 100 µl of substrate [solved in 50 mM Mops buffer (pH 5.5, 6.5, or 7.4)] was added to 100 µl of cells in 96-well flat-bottom plates (no. 655101, Greiner, Monroe, NC). Fluorescence from pDRFlip30 (donor, mCer), pDRFlip39 (donor, t7.ed.eCFP9), and pDRFlip42 linker (donor, mCer) was measured by excitation at λ_exc of 428 nm. Determination of the apparent K_d of NiMet3.0 for NO_3^- was performed as described previously (17). The purified NiMet3.0 protein was pretreated with 0 to 20 mM NO_3^- or 0 to 0.3 mM NO_2^- Data are reported as mean and SD of three to four replicates, and each experiment was performed at least three times with similar results. After 15 min, buffer was exchanged to 50 mM Mops (pH 7.4), and fluorescence was analyzed. The emission ratio was subsequently calculated dividing the value of the 530 nm by 488 nm range.

Expression of NiMet3.0, NiMet3.0-NR-R176A, and nlsNiMet3.0 in Arabidopsis

The p16 promoter (28) from the AT3G60245 gene encoding a 16S ribosomal subunit was used to drive the nuclear-localized NiMet3.0 fusion biosensor, whereas the CaMV35S promoter (56) was used to drive the NiMet3.0 and NiMet3.0-NR-R176A fusion biosensor in plants. The following construct was inserted into the multiple cloning site of the p16-Kan vector (20): 5'-, a sequence coding for the SV40-derived nuclear localization signal LQPKKRRKVVGG (28), a sequence coding for Aphrodite; a Gateway cassette including attrR1, Chloramphenicol resistance gene, ccdB terminator gene, and attrR2; a sequence coding for mCer; and a sequence coding for the cMyc epitope tag -3', or pZPFlip UBQ10-KAN vector under control of the UBQ10 promoter. The resulting Gateway Destination vectors (p16-FLIPnls30 and pZPFlip30) were then recombined in Gateway LR reactions with NasR or NasR-NR-R176A Entry clones, resulting in NiMet3.0, NiMet3.0-NR-R176A, and nlsNiMet3.0 expression clones. Transgenic plant lines were generated using the Agrobacterium floral dip method as described previously (25). Transformants were selected on agar plates containing 1/2 strength MS medium with vitamins (PhytoTech Labs, M519) and with kanamycin.

Fluorescence microscopy

Arabidopsis seedlings were either germinated and grown vertically on 1/2 strength MS agar medium (1/2 strength MS salts without nitrogen; PhytoTech Labs, M531), 1% agar, and 0.05% (w/v) sucrose (pH 5.7) plates or germinated on hydropicnus medium solidified with 1% agar (Becton Dickinson Biosciences) within cut piptette tips, 5 mm in length and 1 mm in diameter, that were positioned in an upright position onto a plate with solidified medium for confocal images or light-sheet images, respectively. Plates were stratified for 3 days at 4°C in the dark before being placed in a growth chamber under long-day growth conditions (16-hour light/8-hour dark cycling, temperature cycling of 22°C day/18°C night, 67% relative humidity). For confocal images, 5- or 6-day-old seedlings were placed in solution containing 1/2 strength MS medium (1/2 strength MS salts without nitrogen and 0.05% sucrose (pH 5.7)) and prepared for imaging on glass slides. Seedlings for light-sheet microscopy were grown for 3 days in the growth chamber, at which time the root tips had almost reached the lower tip outlet. The tips were plugged into a ~3-cm piece of FEP tubing with an inner diameter of 0.115 cm, an outer diameter of 0.195 cm, and wall thickness of 0.04 cm (TEF-CAP, AWG17SW-FEP) and sterilized in 70% ethanol. A closed cultivation system within FEP tubing was used for imaging. Both upper and lower FEP tubes were sealed using gaskets. An inlet and outlet tube were inserted into each side of the gaskets and connected to silicon tubing within a pumping perfusion system. To maintain the humidity within the closed cultivation system, the inner sides of the tubing holder had surrounding water reservoirs. Upon transfer to the light-sheet microscope, the seedling was illuminated by a light connected to a timer switch to maintain the light/dark period. The FEP tubing was filled with 1/20 strength MS hydroponic medium (PhytoTech Labs, M519) and incubated for another 3 to 4 days. The FEP tubing was then fixed in a metal holder and placed into the light-sheet microscope chamber, which was filled with water. The 1/2 strength MS salts hydropicnus medium without nitrogen (pH 5.7) (PhytoTech Labs, M531) was then continually replaced using a peristaltic pump (GE Healthcare) with a flow rate of 1 ml/hour for another 1 to 2 days before the treatments. The temperature of the microscope chamber was set at 22°C.

For NO_3^- treatments on glass slides for confocal microscopy (Figs. 2D, 3, A and C, and 4A and figs. S6 and S7C), seedlings were placed on glass slides with 50 µl of solution and surrounded with a rectangle of vacuum grease and covered with a square coverslip equal in height and half the width of the vacuum grease rectangle. The NO_3^- treatment solution could then be exchanged beneath the coverslip by addition to the left and removal from the right side of the coverslip. Images were acquired at the time points indicated in each figure. Three-dimensional images half the diameter of the primary root axis in Arabidopsis were acquired and analyzed before and after treatments at the time points indicated in the figures or legends (Figs. 2D, 3A, and 4C and D, and figs. S6 and S7C). The central layer image of the primary root axis in plants was acquired
SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abq4915

REFERENCES AND NOTES

1. C. Mascala-Daubresse, F. Daniel-Vedele, J. Dechorgnat, F. Chardon, L. Gaufichon, A. Suzuki, Nitrogen uptake, assimilation and remobilization in plants: Challenges for sustainable and productive agriculture. Ann. Bot. 105, 1141–1157 (2010).

2. N. M. Crawford, B. G. Forde, Molecular and developmental biology of inorganic nitrogen nutrition. Arabidopsis Book 1, e0011 (2002).

3. A. J. Karley, R. A. Leigh, D. Sanders, Where do all the ions go? The cellular basis for sustainable and productive agriculture. Ann. Bot. 105, 1141–1157 (2010).

4. R. Zhang, H. W. Koyro, R. A. Leigh, A. D. Tomas, A. J. Miller, Compartmental nitrate concentrations in barley root cells measured with nitrate-selective microelectrodes and by single-cell sap sampling. Planta 185, 356–361 (1992).

5. H. Zhang, A. Jennings, P. W. Barlow, B. G. Forde, Dual pathways for regulation of root branching by nitrate. Proc. Natl. Acad. Sci. U.S.A. 96, 6529–6534 (1999).

6. P. M. Palenchar, A. Kouranov, L. V. Lejay, G. M. Coruzzi, Genome-wide patterns of carbon and nitrogen regulation of gene expression validate the combined carbon and nitrogen (CN) signaling hypothesis in plants. Genome Biol. 5, R91 (2004).

7. M. Stott, Nitrate regulation of metabolism and growth. Curr. Opin. Plant Biol. 17, 186–189 (2014).

8. D. T. Clarkson, A. Gojon, L. R. Saker, P. K. Wiersema, J. V. Purves, P. Tillard, G. M. Arnold, A. J. M. Paans, W. Vaaulberg, I. Stulen, Nitrate and ammonium influx in soybean (Glycine max) roots: Direct comparison of 15N and 14N tracing. Plant Cell Environ. 19, 859–868 (1996).

9. M. Y. Wang, M. Y. Siddiqui, T. J. Ruth, A. D. M. Glass, Ammonium uptake by root cells (II. kinetics of 15NH4+ influx across the plasmalemma). Plant Physiol. 103, 1259–1267 (1993).

10. I. Guvera, J. Ivanjek, A. Dembiraski-Kiecz, J. Pankiewicz, A. Wanat, P. Anna, I. Golabek, S. Bartusi, M. Malczewska-Malec, A. Szcuddlik, Determination of nitrite/nitrate in human biological material by the simple Griess reaction. Clin. Chim. Acta 274, 177–188 (1998).

11. G. H. Henrikssen, A. J. Bloom, R. M. Spanswick, Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes. Plant Physiol. 93, 271–280 (1990).

12. G. H. Henrikssen, D. R. Raman, L. P. Walker, R. M. Spanswick, Measurement of Net Fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes : II. Patterns of uptake along the root axis and evaluation of the microelectrode flux estimation technique. Plant Physiol. 99, 734–747 (1992).

13. S. Kijomiyi, H. Nakanishi, H. Uchida, A. Tsuji, S. Nishiyama, H. Tsukada, 13N-nitrate uptake sites and rhizobium-biological material by the simple Griess reaction. Clin. Chim. Acta 151, 1743–1753 (2001).

14. H. Matsunami, Y. Arima, K. Watanabe, N. S. Ishioka, S. Watanabe, A. Osa, T. Sekine, S. Matsuhashi, T. Itoh, T. Kume, 13N-translocation in rice under different environmental conditions using positron emitting tracer imaging system. Plant Physiol. 125, 1743–1753 (2001).

15. P. L. Clode, M. R. Kilburn, D. L. Jones, E. A. Stockdale, J. B. Cliff III, A. M. Herrmann, D. V. Murphy, In situ mapping of nutrient uptake in the rhizosphere using nanoscale secondary ion mass spectrometry. Plant Physiol. 151, 1751–1757 (2009).

16. S. Okamoto, A. Jones, W. B. Frommer, Quantitative imaging with fluorescent biosensors. Annu. Rev. Plant Biol. 63, 663–706 (2012).

17. M. Boudes, N. Lazar, M. Graille, D. Durand, T. A. Gaidenko, V. Steward, H. van Tilburg, The structure of the NAsr transcription terminator reveals a one-component system with a NIT nitrate receptor coupled to an ANTAR RNA-binding effector. Mol. Microbiol. 85, 431–444 (2012).

18. W. H. Choi, V. Steward, Nasf, a novel RNA-binding protein, mediates nitrate-responsive transcription antitermination of the Klebsiella oxytoca M5al nasf operon leader in vitro. J. Mol. Biol. 283, 339–351 (1998).

19. J. R. Goodson, C. Zhang, D. Trettel, E. A. Turrell, E. A. Turrell, J. P. E. Lee, C. M. Spirito, W. C. Winkler, An autoinhibitory mechanism controls RNA-binding activity of the nitrate-sensing protein Nasf. Mol. Microbiol. 114, 348–360 (2020).

20. A. M. Jones, J. A. H. Daniels, S. M. Manojkumar, V. V. Lopian, G. Grossmann, W. B. Frommer, Abscisic acid dynamics in roots detected with genetically encoded FRET sensors. eLife 3, e01741 (2014).

21. K. Deuschle, S. Okamoto, M. Fehr, L. L. Looger, L. Kozhukh, W. B. Frommer, Construction and optimization of a family of genetically encoded metallo)m based sensors via semirational protein engineering. Protein Sci. 14, 2304–2314 (2005).

22. A. S. Hires, Y. Zhu, R. Y. Tsien, Optical mapping of synthetic glutamate spillover and reuptake by linker optimized glutamate-sensitive fluorescent reporters. Proc. Natl. Acad. Sci. U.S.A. 105, 4411–4416 (2008).

23. H. Takamaka, B. Chaudhuri, W. B. Frommer, GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. Biochem. Biophys. Acta 1778, 1091–1099 (2008).

24. C. Nathan, Nitric-oxide as a secretory product of mammalian-cells. J. Physiol. 415, 3051–3064 (1992).

25. K. Deuschle, B. Chaudhuri, S. Okamoto, L. Lager, S. Lalande, W. B. Frommer, Rapid metabolism of glucose detected with FRET glucose nanosensors in epidermal cells of intact roots of Arabidopsis RNA-silencing mutants. Plant Cell 18, 2314–2325 (2006).

26. M. T. Madigan, J. M. Martinko, J. Parker, Brock Biology of Microorganisms (Prentice-Hall, 2000).

Chen et al., Sci. Adv. 8, eabq4915 (2022) 19 October 2022
27. A. J. Miller, S. J. Smith, Nitrate transport and compartmentation in cereal root cells. J. Exp. Bot. 47, 843–854 (1996).

28. C. Schuster, C. Gaillochet, A. Medzihradsky, W. Busch, G. Daum, M. Krebs, A. Kehle, J. U. Lehmann, A regulatory framework for shoot stem cell control integrating metabolic, transcriptional, and phytohormone signals. Dev. Cell 28, 438–449 (2014).

29. V. Stewart, Biochemical Society Special Lecture. Nitrate- and nitrite-responsive sensors NarX and NarQ of proteobacteria. Biochem. Soc. Trans. 31, 1–10 (2003).

30. J. Rexach, E. Fernandez, A. Galvan, The Chlamydomonas reinhardtii Nar1 gene encodes a chloroplast membrane protein involved in nitrite transport. Plant Cell 12, 1441–1453 (2000).

31. G. Griffith, M. Sugiuira, M. Takahashi, The function of the plasma-membrane type nitrite transporter (CsNitrl-S) in germinating seeds. Plant Cell Physiol. 48, 538 (2007).

32. M. Sugiura, M. N. Georgescu, M. Takahashi, A nitrite transporter associated with nitrite uptake by higher plant chloroplasts. Plant Cell Physiol. 48, 1022–1035 (2007).

33. M. Takahashi, G. Griffith, M. Sugiuira, A low-affinity nitrite transport of chloroplasts induced by nitrite accumulation in high-affinity nitrite transporter-knockout Arabidopsis mutants. Plant Cell Physiol. 48, 538 (2007).

34. S. Pike, F. Gao, M. J. Kim, S. P. Schachtman, W. Gassmann, Members of the NPF3 transporter subfamily encode pathogen-inducible nitrate/nitrite transporters in grapevine and arabidopsis. Plant Cell Physiol. 55, 162–170 (2014).

35. Z. Kotur, Y. M. Siddiqui, A. D. M. Glass, Characterization of nitrite uptake in Arabidopsis thaliana: Evidence for a nitrite-specific transporter. New Phytol. 200, 201–210 (2013).

36. S. Leran, K. Varala, J.-C. Boyer, M. Chiurazzi, N. Crawford, F. Daniel-Vedele, L. David, S. Pike, F. Gao, M. J. Kim, S. H. Kim, D. P. Schachtman, W. Gassmann, Members of the NPF3 TRANSPORTER family members in plants. Plant Cell Physiol. 55, 162–170 (2014).

37. R. Desikan, R. Griffiths, J. Hancock, S. Neill, A new role for an old enzyme: Nitrate oxidase in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. Proc. Natl. Acad. Sci. U.S.A. 90, 8103–8107 (1993).

38. R. S. Marwaha, B. O. Juliano, Aspects of nitrogen metabolism in the rice seedling. In vivo biochemistry: Applications for small molecule biosensors in plant biology. Curr. Opin. Plant Biol. 16, 389–395 (2013).

39. K. H. Liu, Y. F. Tsay, Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J. 20, 1003–1013 (2001).

40. A. Chamizo-Ampudia, E. Sanz-Luque, A. Llamas, A. Galvan, E. Fernandez, B. Forde, W. Gassmann, D. Geiger, A. Gojon, J.-M. Gong, M. Zhang, R. Dickstein, E. Fernandez, B. Halkier, J. M. Harris, R. Hedrich, A. M. Limami, D. Rentsch, M. Seo, Y.-F. Tsay, M. Zhang, G. Coruzzi, B. Lacombe, A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDETRANSporter family members in plants. Trends Plant Sci. 19, 5–9 (2014).

41. R. Desikan, R. Griffiths, J. Hancock, S. Neill, A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U.S.A. 99, 16314–16318 (2002).

42. R. S. Marwaha, B. O. Juliano, Aspects of nitrogen metabolism in the rice seedling. Plant Physiol. 57, 923–927 (1976).

43. K. H. Liu, Y. F. Tsay, Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J. 22, 1005–1013 (2003).

44. Y.-N. Chen, C.-H. Ho, Concept of fluorescent transport activity biosensor for the characterization of the Arabidopsis NPF1.3 activity of nitrate. Sensors 12, 1198 (2012).

45. A. Chamizo-Ampudia, E. Sanz-Luque, A. Llamas, A. Galvan, E. Fernandez, Nitrate reductase regulates plant nitric oxide homeostasis. Trends Plant Sci. 22, 163–174 (2017).

46. W. M. Kiser, S. C. Huber, Post-translational regulation of nitrate reductase: Mechanism, physiological relevance and environmental triggers. J. Exp. Bot. 52, 1981–1989 (2001).

47. C. MacKintosh, S. E. Meek, Regulation of plant NR activity by reversible phosphorylation, 14–3–3 proteins and proteolysis. Cell. Mol. Life Sci. 58, 205–211 (2004).

48. S. J. Cookson, L. E. Williams, A. J. Miller, Light-dark changes in cytosolic nitrate pools depend on nitrate reductase activity in Arabidopsis leaf cells. Plant Physiol. 138, 1097–1105 (2005).

49. L. Machlis, The respiratory gradient in barley roots. Am. J. Bot. 31, 281–282 (1944).

50. A. Oaks, I. Stulen, K. Jones, M. J. Winspear, S. Misra, I. L. Boesel, Enzymes of nitrogen assimilation in maize roots. Planta 148, 477–484 (1980).