Nitrates Efflux at the Root Plasma Membrane: Identification of an Arabidopsis Excretion Transporter

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ABSTRACT

Root NO$_3^-$ efflux to the outer medium is a component of NO$_3^-$ net uptake and can even overcome influx upon various stresses. Its role and molecular basis are unknown. Following a functional biochemical approach, NAXT1 (for NITRATE EXCRETION TRANSPORTER1) was identified by mass spectrometry in the plasma membrane (PM) of Arabidopsis thaliana suspension cells, a localization confirmed using a NAXT1-Green Fluorescent Protein fusion protein. NAXT1 belongs to a subclass of seven NAXT members from the large NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER family and is mainly expressed in the cortex of mature roots. The passive NO$_3^-$ transport activity ($K_m = 5$ mM) in isolated root PM, electrically coupled to the ATP-dependant H$^+$-pumping activity, is inhibited by anti-NAXT antibodies. In standard culture conditions, NO$_3^-$ contents were altered in plants expressing NAXT1 interfering RNAs but not in naxt1 mutant plants. Upon acid load, unidirectional root NO$_3^-$ efflux markedly increased in wild-type plants, leading to a prolonged NO$_3^-$ excretion regime concomitant with a decrease in root NO$_3^-$ content. In vivo and in vitro mutant phenotypes revealed that this response is mediated by NAXT1, whose expression is upregulated at the posttranscriptional level. Strong medium acidification generated a similar response. In vitro, the passive efflux of NO$_3^-$ (but not of Cl$^-$) was strongly impaired in naxt1 mutant PM. This identification of NO$_3^-$ efflux transporters at the PM of plant cells opens the way to molecular studies of the physiological role of root NO$_3^-$ efflux in stressed or unstressed plants.

INTRODUCTION

Nitrate uptake by plant roots and its subsequent reduction and assimilation are essential for plant growth as well as for N input in many terrestrial trophic chains (Crawford and Glass, 1998; Daniel-Vedele et al., 1998; Williams and Miller, 2001). It results from the balance between an active influx mediated by nH$^+:m$NO$_3^-$ symporters (with $n > m$) and a passive efflux (i.e., an electrically driven uniport) (Crawford and Glass, 1998). Several uptake symporters have been characterized in the NITRATE TRANSPORTER1 (NRT1) and NRT2 gene families (Miller et al., 2007; Tsay et al., 2007), whereas the molecular basis of cellular efflux is still unknown.

In well-supplied and nonstressed plants, NO$_3^-$ efflux can be high but remains lower than influx (Kronzucker et al., 1999), and long-term control of the uptake regime relies on the regulation of active influx transport systems (Lee, 1993). Upon certain biotic (Garcia-Brugger et al., 2006) or abiotic stresses, such as mechanical or transplant shocks (Pearson et al., 1981; Macduff and Jackson 1992; Dehlon et al., 1995; Aslam et al., 1996) or medium acidification (Aslam et al., 1995), marked increases of NO$_3^-$ efflux leading to (net) NO$_3^-$ excretion were reported. The biological significance of this response remains obscure, as does more generally the physiological role of root NO$_3^-$ efflux.

In vitro, it has long been established that the addition of NO$_3^-$ to plasma membranes (PMs) isolated from a wide range of plant and fungal materials strongly stimulates H$^+$-ATPase pumping activity by dissipating the membrane potential ($E_m$) generated by the pump (Vara and Serrano, 1982; Perlin et al., 1984; De Michielis and Spanswick, 1986). This so-called short-circuiting stimulation by NO$_3^-$ provided evidence for the existence of a passive NO$_3^-$ efflux system in isolated PMs. Its functional features indicated that it could be of biological significance, since, in particular, it displays $K_m$ and $V_{	ext{max}}$ values compatible with root NO$_3^-$ cytosolic concentration and efflux capacity, respectively (Pouliquin et al., 2000).

We designed a potentiometric method using the fluorescent oxonol VI dye to measure in vitro both $E_m$ and passive NO$_3^-$ flux on isolated PMs (Pouliquin et al., 1999; Gibrat and Grignon, 2003). On the other hand, we developed methods to solubilize and separate intrinsic membrane proteins in a native (functional) state by gel filtration (GFC) and/or ion-exchange chromatography (IEC). Associated with a final electrophoretic separation in denaturing conditions, GFC/IEC/SDS-PAGE made it possible to identify many membrane transporters by mass spectrometry (MS) (Szponarski et al., 2004, 2007; Delom et al., 2006).

Here, we report the identification of an Arabidopsis thaliana NO$_3^-$ efflux transport protein through a biochemical approach correlating efflux activity and polypeptide abundance in chromatographic fractions of solubilized intrinsic PM proteins from
NAXT1, a Root Plasma Membrane NO₃⁻ Excretion Transporter 3761

RESULTS

A Functional Biochemical Approach to the Identification of a Candidate Protein for PM NO₃⁻ Efflux

A functional biochemical strategy, summarized in Figure 1, was launched on PMs isolated from tobacco (Nicotiana tabacum) BY2 and Arabidopsis suspension cells to identify polypeptide candidate(s) for the NO₃⁻ efflux activity. Intrinsic membrane proteins from BY2 cells were solubilized and separated in native conditions by IEC. In each IEC fraction, image analysis of the SDS-PAGE pattern was performed to determine the abundance of the different polypeptide bands (Figure 1A), and in parallel, the NO₃⁻ efflux activity was measured after reinsertion of the whole protein content into liposomes (Figure 1B). A correlation was then searched for between the abundance of each detected polypeptide band and the activity along successive IEC fractions. Two polypeptide bands of 42 and 17 kD (denoted B₄₂ and B₁₇), both present in the most active fraction, were selected (Figure 1C).

Attempts to obtain sequence data from B₄₂ and B₁₇ by chemical microsequencing were unsuccessful. Since B₄₂ and B₁₇ did not appear to be glycosylated (data not shown), they were used to prepare rabbit polyclonal antisera. Only polyclonal antibodies directed against B₁₇ could ultimately be obtained (A邠Y2). A邠Y2 immunodetected two bands of 17 and 42 kD at a high signal-to-noise ratio and also a faint band at 61 kD in PMs isolated from tobacco BY2 cells (Figure 1D).

Importantly, A邠Y2 provided evidence for the existence of an ortholog of 42 kD in the PM fraction from Arabidopsis suspension cells (Figure 1D). Intrinsic PM proteins from Arabidopsis cells were then separated by GFC/IEC/SDS-PAGE (Figure 1E) (Szponarski et al., 2004, 2007), and MS analysis of the immunodetected B₄₂ led to the identification of an as yet uncharacterized 61-kD protein (NP_190151, encoded from locus At3g45650) herein designated NAXT1. In the presence of a broad-specificity plant protease inhibitor cocktail (AP2 instead of AP1; see Methods), A邠Y2 clearly immunodetected a 61-kD band in the PMs isolated from roots of Arabidopsis (first lane in Figure 4A below). MS analysis of a NAXT1-enriched GFC/IEC/SDS-PAGE fraction of Arabidopsis root PMs confirmed the presence of NAXT1 in the immunodetected 61-kD band (data not shown). This indicated that both the 17- and 42-kD polypeptides previously immunodetected by A邠Y2 were probably proteolytic products derived from the full-length NAXT1 protein.

NAXT1 Belongs to a Subclass of the NRT1/PTR Family

NAXT1 is a highly hydrophobic protein harboring 12 putative transmembrane segments and is predicted to localize in the PM according to PSORT (http://www.psort.org; certainty = 0.8). As illustrated in Figure 2A, this protein belongs to the large NRT1/PTR family (Steiner et al., 1995; Tsay et al., 2007), which encompasses in particular NO₃⁻ uptake transporters. As can be deduced from Figure 2A, NAXT1 and six other NRT1/PTR members constitute a distinct subclass of proteins that we named the NAXT subfamily. NAXT1 isoforms share high similarity with NAXT1 (from 62 to 83% identity at the protein level; see alignment in Figure 2B), and NAXT genes are organized as a cluster on chromosome 3 (The Institute for Genomic Research database; http://www.tigr.org/tdb/e2k1/ath1/TandemDups/duplication_listing.html). It is noteworthy that all members possess a specific 11-amino acid stretch: DINHRVDNVY (the NAXT signature sequence; Figure 2B). Except for At3g45650, whose expression is undetectable in shoots or roots, all NAXT genes are expressed in Arabidopsis roots (Tsay et al., 2007; Arabidopsis eFP Browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; data below). In particular, NAXT1 is preferentially expressed in this organ.

Passive NO₃⁻ Transport Activity at the Isolated Root PM

Root PM was isolated from 6-week-old Arabidopsis plants grown on standard hydroponics, and the activity of the root PM H⁺-ATPase was assayed in vitro in double-labeling experiments with fluorescent probes for ΔpH (9-amino-6-chloro-2-methoxy-acridine [ACMA]) and Eₘ (oxonol VI). Using inside-out membrane vesicles (Grouzis et al., 1997) loaded with 140 mM K⁺ and outer medium without permeant anions, energization of the H⁺-ATPase triggered a slow acidification rate of the vesicle lumen and a large Eₘ (Figures 3A and 3B). The H⁺ pump was slowed by the high (positive inside) Eₘ it created, indicating that the active entry of H⁺ charges was not easily compensated by an equivalent release of K⁺ charges, despite the high K⁺ vesicle content. Addition of the K⁺ ionophore valinomycin triggered a complete depolarization of the vesicles and a high (maximal) H⁺ pumping rate. The short-circuiting effect of valinomycin was not sustained, because vesicles were depleted progressively in K⁺. By contrast (Figures 3C and 3D), addition of a low NO₃⁻ concentration (20 mM) to the outside also depolarized the vesicles, triggering a high and sustained H⁺ pumping rate, because the external NO₃⁻ concentration remained constant. This shows that Arabidopsis root PM contains a highly conductive pathway for NO₃⁻, enabling an Eₘ-driven entry of negative charges associated with NO₃⁻ transport, making Eₘ less positive. Thus, this pathway corresponds to a passive NO₃⁻ transport, which efficiently compensates the active entry of H⁺ charges. Since NO₃⁻ is transported in the same direction as the pumped H⁺, this activity should enable in situ a NO₃⁻ efflux at the root PM. The NO₃⁻-dependent H⁺ pumping rate (ΔVₑₑ) can be taken as an estimate of the equivalent rate of passively transported NO₃⁻ (Vₑₑ). Measurement of initial rates of acidification in the presence of increasing NO₃⁻ concentrations showed that Vₑₑ is saturable, with a Michaelis constant of 5 mM (Figure 3E).
Figure 1. Biochemical Strategy That Led to the Identification of NAXT1.

The biochemical strategy was developed on PMs isolated first from tobacco BY2 cells and second from *Arabidopsis* suspension cells. After PM stripping and nondenaturing solubilization (see Methods), intrinsic proteins from tobacco BY2 cells were rapidly fractionated by IEC using various exchanger columns and salt gradients. Polypeptide abundance was estimated in successive IEC fractions by image analysis of SDS-PAGE patterns. NO$_3^-$ efflux and permeability coefficients were determined in parallel by imposing K$^+$ diffusion $E_{m}$ on proteoliposomes reconstituted from protein fractions. Data from different fractionation experiments are expressed on a relative basis and merged in order to search for a correlation between the facilitated passive transport activity and abundance of the different polypeptide bands detected by SDS-PAGE. Two tobacco polypeptides of 42 and 17 kD (denoted B$_{42}$ and B$_{17}$, respectively) were correlated, but attempts to obtain sequence data after excision and trypsin digestion of the bands were unsuccessful. B$_{17}$ was successfully used to prepare rabbit polyclonal antibodies (A$_{BY2}$), which made it possible to immunodetect essentially two polypeptides of 17 and 42 kD in PMs from both tobacco and *Arabidopsis* cells. After fractionation by GFC/IEC/SDS-PAGE, the immunodetected B$_{42}$ of *Arabidopsis* was excised and the trypsin-digested peptides were analyzed by MS, leading to the identification of NAXT1. FPLC, fast protein liquid chromatography.
The NAXT Subfamily Is Involved in NO₃⁻ Efflux Activity in Vitro and NO₃⁻ Distribution in Plants

Attempts to obtain NAXT1-specific antibodies were unsuccessful due to the high similarity between NAXT members and their high sequence hydrophobicity (Figure 2B). Therefore, rabbit polyclonal antibodies potentially directed against all NAXT members were prepared from a mixture of three NAXT1-derived synthetic peptides (P1, P2, and P3; Figure 2B) and further immunopurified against the peptides. The resulting polyclonal antibodies (ANAXT) detected the same 61-kD band in Arabidopsis root PMs detected previously with ABY2 (Figure 4A).

The ability of ABY2 and ANAXT antibodies to specifically inhibit the NO₃⁻ efflux activity in vitro (VNO₃) was tested using a previously established functional assay (Gaymard et al., 1993). As a positive control, polyclonal anti-PM ATPase antibodies (Santoni et al., 1993), designated here ANAXT. Inhibited by >80% the maximum rate of H⁺ excretion by the H⁺-ATPase short-circuited with the K⁺ ionophore valinomycin (Figure 4B). Conversely, as expected, this activity was not affected significantly by ANAXT at an identical 5:1 IgG:protein ratio. In the presence of ANAXT and ABY2 at a 1:1 IgG:protein ratio, VNO₃ decreased by 60 and 45%, respectively (Figure 4C), whereas no inhibition was observed with preimmune antibodies (APh). In the presence of ANAXT and ABY2 at a 5:1 IgG:protein ratio, inhibition levels reached 85 and 70%, respectively, relative to VNO₃ in the presence of APh. This indicates that most of the NO₃⁻ efflux activity in root PM vesicles is mediated by NAXT proteins.

RNA interference (RNAi) transgenic plants (reviewed in Brodersen and Voinnet, 2006; Mansoor et al., 2006) impaired in the expression of all NAXT members (naxt-RNAi) were obtained (Figures 5A and 5B). Although no growth phenotype was observed, NO₃⁻ contents of naxt-RNAi plants grown in vitro on standard Murashige and Skoog medium were altered significantly compared with those of wild-type plants (30% higher in roots and 26% lower in shoots; Figure 5C). This indicates that one or several NAXT members are involved in the distribution of NO₃⁻ in plants.

NAXT1 Is a PM Transporter Expressed Mainly in Root Cortex

Transient expression of a P35S:NAXT1-GFP (for Green Fluorescent Protein) fusion construct in Arabidopsis protoplasts revealed the localization of the NAXT1-GFP fusion protein in the PM (Figure 6), consistent with the previous identification of the NAXT1 protein in a purified PM fraction.

NAXT1 gene expression was stronger in roots than in shoots (Figure 7A). Localization of NAXT1 expression in root tissues was investigated using transgenic Arabidopsis plants expressing PNAXT1:GUS fusion constructs harboring the β-glucuronidase (GUS) gene under the transcriptional control of the NAXT1 promoter. Because of the small size of the upstream intergenic region (563 nucleotides), short and long versions of the NAXT1 promoter sequence (PNAXT1) were used, 563 and 1029 nucleotides upstream of the NAXT1 open reading frame (ORF) ATG, respectively. In 7-d-old seedlings expressing the GUS reporter gene, no difference in GUS patterns was observed between the two types of constructs (data not shown). This suggests that the 563-nucleotide PNAXT1 sequence contained all of the necessary information for NAXT1 tissue localization.

Analysis of GUS staining was performed at three developmental stages on 7-d-old (Figure 7B), 14-d-old (Figure 7C), and 6-week-old (Figures 7D and 7E) plants. Although cotyledons were stained to some extent, most of the staining was found in the mature root zone. In roots of 6-week-old plants grown in hydroponics (like those used in the naxt1 phenotyping experiments), diminished staining was observed in the youngest part of the primary root, while secondary roots and younger parts of the primary root were still strongly stained (Figure 7D). This may reflect the fact that young roots are generally considered to have higher nutrient transport activity than old roots (Gao et al., 1998). Root tips were not stained (Figure 7E), and GUS staining in the mature root was observed mainly in cortical cells and to a lesser extent in epidermal cells (Figures 7F and 7G).

These results show that NAXT1 is essentially expressed in the mature root cortex and are consistent with the hypothesis that NAXT1 is involved in root NO₃⁻ excretion to the external medium.

tnax1 T-DNA Insertion Mutants Grown in Standard Conditions Do Not Display an Apparent Phenotype

Light and plant N status, both known to regulate the gene expression of major NO₃⁻ uptake transporters such as NRT1:1 (Forde, 2000), did not affect NAXT1 gene expression in hydroponically grown plants (see Supplemental Figure 1 online). Phenotypes resulting from the alteration of NAXT1 gene expression were searched for in standard culture conditions using naxt1-1 and naxt1-2, two T-DNA insertion lines (insertions in exon 3 of NAXT1) in which the mRNA of NAXT1 was undetectable (see Supplemental Figures 2A and 2B online). Wild-type and naxt1 plants displayed no significant difference in root and shoot...
Figure 2. NAXT1 Belongs to a Subclass of the NRT1/Ptr Family.

(A) Unrooted phylogenetic tree of Arabidopsis proteins of the NRT1/Ptr family. Members for which functional characterization is described in the literature are indicated in boldface. The NAXT protein subclass is highlighted in the gray oval.

(B) Multiple sequence alignment of the seven NAXT proteins. Black-boxed residues are shared by at least four NAXT members. The consensus sequence showing residues conserved in all NAXT members is presented below each line of the alignment. The positions of the NAXT1-derived P1, P2, and P3 peptide sequences used to generate the ANAXT antibodies are indicated. The 11–amino acid long NAXT signature sequence is boxed.
growth (data not shown), in root and shoot NO$_3^-$ contents (Figure 3C; controls in Figures 8B and 10A below), in unidirectional root NO$_3^-$ efflux and influx (Figure 8), and in passive NO$_3^-$ transport activity in vitro (controls in Figures 9 and 10 below). Consequently, although some NAXT members are involved in passive NO$_3^-$ transport and in root/shoot NO$_3^-$ distribution, the contribution of NAXT1 seems negligible in plants grown in standard conditions.

NAXT1 Is the Efflux Transporter Responsible for the Root NO$_3^-$ Excretion Induced by Acid Load

It was hypothesized that the absence of an apparent transport phenotype was the result of inappropriate phenotyping conditions. Previous studies had revealed that in isolated root PMs, NO$_3^-$ efflux is tightly coupled to H$^+$ excretion by the H$^+$-ATPase (Vara and Serrano, 1982; De Michielis and Spanswick, 1986; Grouzis et al., 1997) and that both NO$_3^-$ efflux and H$^+$ excretion activities share similar acidic optimum pH at the cytosolic face of the PM (Pouliquen et al., 2000). Also, in vivo stimulation of H$^+$ excretion upon acid load is a well-established feature of the PM H$^+$-ATPase (Hager and Moser, 1985; Mathieu et al., 1986; Beffagna and Romani 1991). In search for a phenotype in naxt1 mutant plants, the assumption that acid load could also stimulate root NO$_3^-$ efflux was addressed experimentally. Using the $^{15}$N isotope, unidirectional root NO$_3^-$ efflux and influx ($F_{\text{co}}$ and $F_{\text{oc}}$, respectively) were determined on the same plants as described previously (Delhon et al., 1995). Propionate, a permeant weak acid widely used for plant cell acid loading (Felle, 2005), was

Figure 3. Nitrate Efficiently Abolishes the $E_m$ Generated by the H$^+$-ATPase of Arabidopsis Root PM.
added to the plant nutrient medium (10 mM final concentration). The cytosolic pH is known to decrease within a few minutes after the addition of propionate (Tournaire-Roux et al., 2003), but preliminary tests indicated that longer times of acid load were required to reveal a naxt1 phenotype. In wild-type roots, \( \Phi_{oc} \) was 50% higher after 30 min of acid load, compared with unloaded controls (Figure 8A). This increase was abolished in the naxt1-1 mutant.

Unexpectedly, \( \Phi_{oc} \) largely exceeded \( \Phi_{co} \) in wild-type and naxt1 unloaded controls (Figure 8A, inset), showing that even control plants underwent a \( \text{NO}_3^-/\text{CO}_2 \) excretion regime. However,

Figure 4. \( \text{ABY}2 \) and \( \text{ANAXT} \) Antibodies Inhibit PM \( \text{NO}_3^-/\text{CO}_2 \) Efflux Activity in Vitro.

(A) \( \text{ABY}2 \) and \( \text{ANAXT} \) antibodies immunodetect the same 61-kD NAXT band in \( \text{Arabidopsis} \) root PM proteins. The protease inhibitor cocktail AP2 (see Methods) was used throughout PM isolation.

(B) Negative and positive controls performed on PM \( \text{H}^+\text{-ATPase} \) activity. The maximum \( \text{H}^+\text{-pumping} \) activity (\( V_{\text{H}}^+ \)) was measured in the presence of valinomycin. Antibodies directed against PM \( \text{H}^+\text{-ATPase} \) (\( \text{AATPase} \)) efficiently inhibit the \( \text{H}^+ \) pumping activity, whereas \( \text{ANAXT} \) does not. The IgG:protein ratio was 5:1 (w/w). Ct, control treatment (no IgG added).

(C) Inhibition of \( \text{NO}_3^-/\text{CO}_2 \) efflux activity. \( \text{ABY}2 \) and \( \text{ANAXT} \) antibodies efficiently inhibit \( \text{NO}_3^- \) efflux activity (\( V_{\text{NO}_3^-} \)) assayed at 20 mM \( \text{NO}_3^- \) at the indicated IgG:protein ratios (w/w). PI, preimmune.

Error bars in (B) and (C) indicate SE (\( n = 3 \)).

Figure 5. Root and Shoot \( \text{NO}_3^-/\text{CO}_2 \) Contents of Plants Grown in Vitro in Standard Conditions: Effect of the Suppression of the Expression of NAXT Genes.

(A) Schematic representation of the RNAi cassette integrated in the genome of naxt-RNAi transgenic plants. The 296-bp-long NAXT1 ORF cDNA fragment (nucleotides 457 to 753) displays 80 to 93% identity to corresponding sequences of other NAXT members. This cDNA fragment was cloned in the pHannibal vector in the two opposite orientations (arrows) downstream and upstream of the PDK intron (see Methods). nt, nucleotide; PDK, pyruvate orthophosphate dikinase; Pro 35S, 35S promoter from \( \text{Cauliflower mosaic virus} \); Ter OCS, octopine synthase terminator.

(B) RT-PCR analysis of total root RNAs shows at least partial suppression of gene expression of all NAXT members in naxt-RNAi plants. The expression level of \( \text{Elongation Factor} \, (EF1\alpha) \) was used as an internal control. PCR was performed using 28 cycles, except for At3g45720 (35 cycles) and \( EF1\alpha \) (20 cycles). At3g45690 was undetectable in either plant even using 35 PCR cycles (data not shown). Primer sequences are provided in Supplemental Table 1 online.

(C) In standard conditions, naxt1-1 mutant plants display wild-type-like root and shoot \( \text{NO}_3^-/\text{CO}_2 \) contents, but naxt-RNAi plants show significantly altered \( \text{NO}_3^- \) contents in both roots and shoots. The T-DNA insertion line naxt1-1 is described in Supplemental Figure 2 online. DW, dry weight.
root NO$_3^-$ contents measured prior to, and 30 min after, plant transfer in the $^{15}$N assay medium were similar, indicating that the excretion regime was transient in the absence of acid load (Figure 8B). This transient excretion is likely attributable to the transplant shock subsequent to the transfer of plants to the $^{15}$N assay medium performed just prior to $F_{co}$ and NO$_3^-$ measurements (Delhon et al., 1995). Indeed, mechanical stress almost instantaneously triggers a NO$_3^-$ excretion due to a strong increase in the efflux, which recovers its basal value within a few minutes, the influx being unaffected (Aslam et al., 1996).

Here, NAXT1 does not seem to be involved in the transient excretion observed after plant transfer, because $F_{co}$ and NO$_3^-$ contents of wild-type and naxt1-1 roots were very similar in unloaded controls.

By contrast, 30 min after the addition of propionate to the nutrient medium (containing 1 mM NO$_3^-$), the root NO$_3^-$ content decreased by 30% in the wild type, whereas it was not affected in either the naxt1-1 or the naxt1-2 mutant (Figure 8B). Furthermore, it decreased by 45% in a naxt1-1 mutant complemented with the NAXT1 coding sequence expressed constitutively under the control of the cauliflower mosaic virus (CaMV) 35S promoter harboring a duplicated enhancer sequence (P70) (Kay et al., 1987). Thus, acid load is responsible for prolonged root NO$_3^-$ excretion mediated by NAXT1, leading to a marked decrease in root NO$_3^-$ content. This is the reason for the designation of this protein as NAXT (for NITRATE EXCRETION TRANSPORTER).

Prolonged acid load could not be easily used in vivo to investigate its effect on $F_{co}$, because of the large and rapid decrease in root NO$_3^-$ content (i.e., in NO$_3^-$ potentially available to sustain $F_{co}$). Therefore, NO$_3^-$ efflux activity in vitro ($V_{NO3}$) was measured on root PM isolated from plants acid-loaded (or not) for 1 or 3 h. $V_{NO3}$ in wild-type, naxt1-1, and naxt1-2 controls (non-acid-loaded plants) was indistinguishable (Figure 9A), consistent

Figure 6. The NAXT1-GFP Fusion Protein Is Expressed in the PM of Arabidopsis Cells.

GFP constructs were expressed transiently in Arabidopsis protoplasts, and signals were examined with a laser confocal microscope under transmitted light ([A] and [C]) or under fluorescence after excitation at 530 nm ([B] and [D]). Bars = 10 μm. (A) and (B) Protoplast transformed with the control construct (P35S:GFP).

(C) and (D) Protoplast transformed with pG611, a construct expressing NAXT1 fused to the N-terminal part of GFP (P35S:NAXT1-GFP). T, tonoplast.

Figure 7. NAXT1 Is Expressed Mainly in the Cortex of Mature Roots.

(A) The NAXT1 gene is more strongly expressed in roots than in leaves. RT-PCR was performed on 6-week-old hydroponic plants. The two primers used were F1-738 and R1-1457 (sequences provided in Supplemental Table 1 online). The expression of EF1α was used as a control.

(B) to (G) Histochemical analyses of transgenic Arabidopsis plants expressing the PNAXT1:GUS construct. Plants were cultured in vitro ([B], [C], [F], and [G]) or in hydroponics ([D] and [E]). (B) and (C) Micrographs of 7-d-old (B) and 14-d-old (C) seedlings showing major GUS staining in roots. Bar = 1.2 mm.

(E) No GUS staining is observed at the root tip.

(F) Micrograph of the mature root of a 7-d-old seedling showing strong GUS staining in the cortex. Bar = 50 μm.

(G) Cross section of the mature root of a 7-d-old seedling showing stained cortical cells. The epidermis is also stained, but less strongly. Bar = 50 μm.
with the absence of an efflux phenotype observed previously in planta.

By contrast, \( V_{\text{NO}_3} \) was higher by 50 and 80% in wild-type PM after 1 and 3 h of acid load, respectively. This increase was impaired in both \( naxt1-1 \) and \( naxt1-2 \) mutants, showing that the increase in \( V_{\text{NO}_3} \) observed in wild-type PM upon acid load resulted from the induction of a NAXT1-mediated activity.

**NAXT1 Expression Is Regulated at the Posttranscriptional Level**

The acid load–mediated functional response described above was not related to an upregulation of \( NAXT1 \) gene expression, because the \( NAXT1 \) transcript level remained stable upon acid load of wild-type roots (Figure 9B). Since no \( NAXT1 \)-specific antibodies were available to directly investigate \( NAXT1 \) protein level in the wild type, protein gel blot signals of root PM from the wild type and \( naxt1 \) mutants were compared using \( A_{\text{NAXT}} \). No significant difference was observed between these plants when grown in standard conditions (Figure 9C), in agreement with the absence of a mutant phenotype reported above. By contrast, the intensity of the NAXT band increased significantly after 1 h of acid load in the wild type but not in the two \( naxt1 \) mutants, suggesting that \( NAXT1 \) accumulates in the wild-type PM upon acid load.

The intensity of the NAXT band also increased in the whole membrane fraction (P100K) upon acid load (Figure 9D), showing that the \( NAXT1 \) accumulation in the PM is unlikely to result from its relocalization from another storage membrane compartment. The acid load–dependent accumulation of \( NAXT1 \) in the wild-type PM was then monitored in transformed plants constitutively expressing the \( NAXT1 \) coding sequence under the control of the P70 promoter (Figure 9E). In complemented \( naxt1-1 \) plants, the \( NAXT \) band (detected by \( A_{\text{NAXT}} \)) increased after acid load, suggesting that the accumulation of \( NAXT1 \) transcript and protein is uncoupled. In a transgenic line constitutively expressing \( NAXT1 \) tagged with the AcV5 epitope (Lawrence et al., 2003), the \( NAXT1\)-AcV5 fusion protein (detected by a monoclonal anti-AcV5 antibody) also accumulated after acid load. This demonstrates that the expression of \( NAXT1 \) is regulated at the posttranscriptional level.

**NAXT1 Is the Efflux Transporter Responsible for the Root \( \text{NO}_3^- \) Excretion Induced by Plant Medium Acidification**

Medium acidification can be considered a biologically more relevant treatment than acid load artificially generated with propionate. Acidification of the barley (\( Hordeum vulgare \)) root hydroponic medium was shown to have little effect on \( \text{NO}_3^- \) influx but markedly stimulated efflux (Aslam et al., 1995). In particular, a net excretion regime was observed in 2 mM MES buffer at pH 3. We hypothesized that medium acidification could be responsible for an acidic drift of the cytosolic pH and induce a response similar to that observed upon acid load. Indeed, membrane bilayers display a surprisingly high permeability coefficient to \( H^+ \) (\( \sim 10^{-6} \text{ M}^{-1} \text{ s}^{-1} \); Rossignol et al., 1982). Moreover, accounting for the large (negative) \( E_m \) of root cells, a strong passive entry of \( H^+ \) within root cells is expected at pH 3.

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**Figure 8.** \( NAXT1 \) Is Required for the Acid Load–Dependent Root \( \text{NO}_3^- \) Excretion.

Acid load was applied on 6-week-old plants grown on standard hydroponics by adding 10 mM propionate to the medium 30 min prior to the determination of root \( \text{NO}_3^- \) content or prior to plant transfer to measure unidirectional \( \text{NO}_3^- \) influx (\( \Phi_{\text{OC}} \)) and efflux (\( \Phi_{\text{CO}} \)). DW, dry weight. (A) The increase of \( \Phi_{\text{CO}} \) in the wild type is abolished in \( naxt1-1 \) plants. The inset shows \( \Phi_{\text{OC}} \) in the same plants. Error bars indicate SE (\( n = 8 \)). (B) The decrease in root \( \text{NO}_3^- \) content observed in wild-type plants after acid load (gray bars) is abolished in \( naxt1 \) mutants and restored in the complemented \( naxt1-1 \) mutant. Control plants (no acid load) were transplanted (white bars) or not (black bars) prior to the determination of root \( \text{NO}_3^- \) content. Error bars indicate SE (\( n = 6 \)).
Consistently, wild-type root NO$_3^-$ content decreased by almost 50% after acidification of the plant nutrient medium for 30 min at pH 3, whereas it was not affected significantly in either naxt1-1 or naxt1-2 mutant roots (Figure 10A). NAXT1 expression remained stable upon medium acidification, whereas protein gel blot analyses showed a marked increase in the intensity of the NAXT band in wild-type but not in naxt1-1 root PM (Figure 10B). These data indicate that, like acid load, medium acidification induces prolonged root NO$_3^-$ excretion mediated by NAXT1, which is also upregulated at the posttranscriptional level.

Some intrinsic properties of the NO$_3^-$ efflux were also determined on root PM of plants treated at pH 3, using a transport assay independent of the H$^+$/ATPase (Pouliquin et al., 1999). As detailed in Supplemental Figure 3 online, the rate of passive NO$_3^-$ flux ($J_{\text{NO}_3}$) was determined from the rate of NO$_3^-$-dependent dissipation of imposed diffusion $E_m$ monitored quantitatively with oxonol VI. $J_{\text{NO}_3}$ in wild-type PM, assayed between pH 5.5 and 7.0, revealed an optimal pH at 6.5 (Figure 10C). $J_{\text{NO}_3}$ in naxt1-1 was negligible compared with $J_{\text{NO}_3}$ in the wild type at pH 6.5 (Figure 10D), showing that, essentially, NAXT1 operates at this optimal acidic pH. By contrast, naxt1-1 was not affected significantly in passive Cl$^-$ flux, showing that NAXT1 is not competent for Cl$^-$.  

**DISCUSSION**

**A Biochemical Strategy for the Identification of Secondary Transport Systems**

The plant PM H$^+$-ATPase, an archetype of the plant primary transporter, is one of the most electrogenic pumps, able to generate $E_m$ down to –250 mV (Sanders and Slayman, 1989). Here, the H$^+$ pump from *Arabidopsis* PM is short-circuited in vitro after the addition of a low concentration of NO$_3^-$ (20 mM), as already observed in maize (*Zea mays*) root PM (Grouzis et al., 1997). By contrast, short-circuiting of the H$^+$ pump by K$^+$ requires the addition of a K$^+$ ionophore and 10-fold higher K$^+$ concentrations. In vitro, the root cell PM appears more conductive to NO$_3^-$ than to K$^-$. This conclusion is consistent with the large permeability coefficient to NO$_3^-$ observed in vesicles from maize root PM ($P_{\text{NO}_3} \approx 10^{-9} \text{ M}^{-1} \text{s}^{-1}$; Pouliquin et al., 1999).
These observations made in vitro have implications in terms of the relative abundance of transport systems at the surface of plant cells, because isolated PM fractions are constituted of microscopic vesicles. After cell homogenization, 1 μm² of PM surface generates ~100 vesicles (Pouliquin et al., 2000). PM H⁺-ATPase accounts for ~5% of root PM proteins (Serrano, 1985; Sussman, 1994), which would approximately correspond to 500 H⁺ pump molecules per 1 μm². Therefore, it is expected that most PM vesicles are competent for H⁺ pumping. Similarly, the NO₃⁻ short-circuiting of the H⁺ pump observed in PM vesicles indicates that most vesicles are also competent for passive NO₃⁻ transport and thus that the transport is mediated by a relatively abundant PM transport protein. From these considerations, it was anticipated that such a transport system should be amenable to biochemical tracking based on an abundance versus activity correlation approach in native chromatographic fractions of intrinsic PM proteins, followed by MS identification of candidate proteins. This strategy led here to the identification of NAXT1, a transporter belonging to the large NRT1/PTR family.

Two types of antibodies were obtained in this study. Antibodies were directed against a candidate polypeptide band correlated with the efflux activity in chromatographic fractions of PM proteins from tobacco. Antibodies were raised against synthetic peptides to specifically detect NAXT homologs in Arabidopsis. Despite differences in their conception and origin, both antibodies immunodetected the same NAXT-containing 61-kD polypeptide band in Arabidopsis root PM and both specifically inhibited NO₃⁻/C₀ efflux activity measured in vitro. These results validate the candidate protein identified following the biochemical approach adopted here.

This study demonstrates the feasibility of a functional biochemical strategy to identify ion transporters. However, such an approach is probably not suitable for the identification of transport systems displaying poorly detectable activity at the isolated PM level and/or very low abundance. Ion channels, for example, are poorly abundant in membranes from plant tissues (typically, from 0.1 to 1 channel molecule per 1 μm²). Despite their high molecular transport activity, they would functionally remain almost undetectable at the vesicle level, since only one vesicle per 100 to 1000 should be channel-competent. Consistently, a K⁺ ionophore is required to short-circuit H⁺-ATPase in isolated root PM vesicles, although different K⁺ channels coexist in this membrane.

A Molecular Basis for Passive NO₃⁻ Efflux Activity at the PM of Plant Cells

Nitrate transport activity coupled to the PM H⁺-ATPase was long since demonstrated in vitro on root PM vesicles (Vara and Serrano, 1982; Perlin et al., 1984; De Michielis and Spanswick, 1986). Using antibodies directed against NAXT proteins (A₅Ax) in a functional inhibition assay, we show here that NAXT transporters are responsible for most of this activity in Arabidopsis root PM.

In vitro, two different spectroscopic methods applied to membrane vesicles made it possible to show that NAXT-mediated NO₃⁻ transport is driven by Eₘ and associated with the transfer
of a net negative charge in the same direction. NAXT nH\(^+\):mNO\(_3^-\) cotransport mechanisms with m > n seem unlikely in the light of the comparison of the maximum acidification rates of the H\(^+\)-ATPase short-circuited by valinomycin or by NO\(_3^-\) (Figure 3). Valinomycin is an ionophore mediating a passive K\(^+\) uniport, expected to enable the exchange of one K\(^+\) per pumped H\(^+\). Therefore, valinomycin-mediated K\(^+\) short-circuiting allows for the measurement of the true maximum initial acidification rate of the H\(^+\)-ATPase. The initial acidification rate in the presence of 60 mM NO\(_3^-\) accounted for 85\% of the value measured in the presence of valinomycin. If NO\(_3^-\) was transported according to an antiport or a symport mechanism, significantly lower or higher maximum initial acidification rates, respectively, should have been observed upon NO\(_3^-\) short-circuiting. Thus, the NAXT system likely mediates an E\(_m\)-driven uniport of one NO\(_3^-\) per H\(^+\) pumped by the H\(^+\)-ATPase. In situ, the NAXT system is expected to mediate a passive NO\(_3^-\) efflux at the root PM, because it is functionally coupled to the root PM H\(^+\)-ATPase and NO\(_3^-\) is transported in the same direction as the pumped H\(^+\). This conclusion is consistent with physiological studies indicating that root NO\(_3^-\) efflux, in contrast with NO\(_3^-\) influx, is a thermodynamically downhill process (reviewed in Crawford and Glass, 1998; Miller et al., 2007). Moreover, although the extent of the cytosolic NO\(_3^-\) pool is debated (Britto and Kronzucker, 2003), the K\(_m\) value of the NAXT system observed in vitro is consistent with the range of cytosolic NO\(_3^-\) values recently observed by different methods in well-supplied plants (Radcliffe et al., 2005; Ritchie, 2006).

Except for At3g45690, whose expression was undetectable in shoots or in roots, all NAXT genes are expressed in Arabidopsis roots (Tsay et al., 2007; Arabidopsis eFP Browser, http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi; this work) and several NAXT proteins have been identified in wild-type root PM by MS (W. Szonarski, personal communication). The strong similarity between the NAXT proteins should be emphasized and suggests a recent duplication event from a common ancestor gene. Although it cannot be specified at present which members of the NAXT family actually contribute to the NO\(_3^-\) efflux activity in root PM from plants grown in standard hydroponics, it is likely that all members share a common transport function in PM.

In planta, the \(^{15}\)N method used here allowed the measurement of both root unidirectional efflux and influx (\(\Phi_{co}\) and \(\Phi_{oc}\)) on the same plants. This made it possible to show that NAXT1 is responsible for an acid load–induced increase in \(\Phi_{co}\), which reaches a value 15-fold higher than \(\Phi_{oc}\) in vitro, using two different spectroscopic methods, the increase in passive NO\(_3^-\) efflux measured in the PM from plant roots subjected to acid load or medium acidification was found to be mediated by NAXT1.

This study provides a molecular basis for passive anion efflux at the PM of plant cells and introduces a new transport function in the large NRT1/PTR family (~50 members). To date, few Arabidopsis NRT1/PTR members have been characterized at the functional level (Tsay et al., 2007). Among them, At NRT1 proteins are involved in NO\(_3^-\) uptake (three members characterized to date: Huang et al., 1999; Liu et al., 1999; Chiu et al., 2004), whereas At PTR proteins are involved in oligopeptide uptake (three members characterized: Song et al., 1996; Dietrich et al., 2004; Karim et al., 2005). In addition, eight other Arabidopsis NRT1/PTR members were proven to encode NO\(_3^-\) transporters after their heterologous expression in Xenopus oocytes, according to unpublished data mentioned by Tsay et al. (2007).

The finding that passive NO\(_3^-\) efflux can be mediated by a subclass of NRT1/PTR transporters was rather unexpected with regard to the variety of channels mediating the passive transport of mineral ions in plants. In particular, various anion channels have been functionally characterized in roots, with different selectivities, gating, and pharmacological signatures (reviewed in Roberts, 2006). In isolated PM vesicles, passive NO\(_3^-\) efflux was shown to be voltage-gated, and data were formally equivalent to a current mediated by a rectifying anion channel displaying an optimum voltage at ~105 mV (Pouliquin et al., 2000). Nevertheless, transporters can also be voltage-gated, as exemplified by the 1 NO\(_3^-\):2 H\(^+\) symport activity observed upon expression of the high-affinity active fungal transporter NrtA in Xenopus oocytes (Boyd et al., 2003). Furthermore, the prokaryotic CLCec anion channel, displaying an almost occluded aqueous pore, was shown to mediate a stoichiometrically fixed anion/proton antiport activity (reviewed in Miller, 2006). Such a transporter activity involving channel-like features was also recently reported for the plant vacuolar At CLCa (De Angeli et al., 2006). These data indicate that the distinction between channels and transporters is not clear-cut, although ion channels generally display high transport activities and low abundances relative to transporters. It is worth noting that anion channels could also be involved in the root NO\(_3^-\) efflux observed in planta, but their activity would likely remain undetectable in membrane vesicle preparations, as discussed above.

Finally, it should be emphasized that since all NAXT members are expressed in root tissues, confirmation of the molecular transport mechanisms and properties of the different members will require their functional analysis in a suitable heterologous system.

NAXT1 Protein Accumulation in PM and naxt1 Mutant Phenotypes

In standard culture conditions, naxt1 mutants displayed no apparent phenotype, although NAXT1 gene expression was detected and NAXT1 protein was identified by MS in wild-type root PM. In particular, PM isolated from wild-type and naxt1 roots displayed similar NO\(_3^-\) efflux activities. Since the intensity of the immunodetected NAXT band was not significantly affected by the disruption of the NAXT1 gene, it is likely that the NAXT1 protein accounts for only a minor part of the pool of NAXT proteins in the root PM of wild-type plants grown in standard conditions.

Acid load or medium acidification did not affect the NAXT1 transcript level. However, both induced an increase in the intensity of the immunodetected NAXT band in wild-type but not in naxt1 mutant root PM. This suggests that, essentially, the NAXT1 protein accumulates in wild-type PM upon these treatments and that this accumulation strongly increases the pool of NAXT proteins. The acid load–induced accumulation of a constitutively expressed AcV5-tagged NAXT1 protein confirmed that NAXT1 accumulation in the PM is controlled at the posttranscriptional level.
To our knowledge, two other examples of the uncoupling of mRNA and protein accumulation have been described for ion transporters of Arabidopsis PM, using similar 3SS transgenic plants. When the high-affinity iron uptake transporter IRT1 (Connolly et al., 2002) or the boron exporter BOR1 (Takano et al., 2005) are constitutively expressed in transgenic lines under the control of the CaMV 3SS promoter, both transporters accumulate in limiting substrate conditions but are degraded upon substrate resupply. In the presence of sufficient substrate, the turnover of IRT1 is proposed to be downregulated by ubiquitin-dependent proteolysis, whereas BOR1 is transferred to the vacuole via the endosomes for degradation.

Here, a posttranscriptional control was found to limit the PM accumulation level of NAXT1 in standard hydroponics. It is noteworthy that \( \text{NO}_3^- \) efflux increases the energetic cost of the net \( \text{NO}_3^- \) uptake, since a minimum expenditure of 2 mol of ATP is required per mole of absorbed \( \text{NO}_3^- \) (Briskin et al., 1995). Nitrate efflux has been reported to be responsible for higher respiratory costs of \( \text{NO}_3^- \) uptake in slow-growing grasses (Scheurwater et al., 1999). Furthermore, induction of NAXT1 accumulation was found here to promote a net excretion regime that is expected to have strong detrimental effects for N nutrition. In this context, the posttranscriptional control limiting the NAXT1 accumulation level in standard culture conditions seems essential.

Posttranscriptional mechanisms allowing variable expression of a protein while the mRNA remains constantly expressed are expected to ensure rapid functional responses. Consistently, \text{naxt1}\#_mutant plants display a clear root \( \text{NO}_3^- \) excretion phenotype after 30 min of acid load or medium acidification. This could reveal the importance of \( \text{NO}_3^- \) excretion, an energetically costly process consuming a nutritional resource, as an early response to marked acidic drift of the cytosolic pH. Because of the tight coupling of \( \text{H}^+ \) and \( \text{NO}_3^- \) excretion observed in vitro (Vara and Serrano, 1982; De Michielis and Spanswick, 1986; Grouzis et al., 1997) and the known abundance of acid load to stimulate \( \text{H}^+ \) excretion by PM ATPase in vivo (Hager and Moser, 1985; Mathieu et al., 1988; Beffagna and Romani 1991), we hypothesized that cytosolic acidification of root cells leads to \( \text{NO}_3^- \) excretion (Pouliquen et al., 2000). The results obtained here support this hypothesis. In particular, NAXT1 displays optimal activity at pH 6.5. However, the biological significance of such \( \text{NO}_3^- \) excretion remains unclear. In principle, \( \text{H}^+ \) excretion by \( \text{H}^+ \text{-ATPase} \) is expected to attenuate acidic drift in the cytosol, provided excreted \( \text{H}^+ \) can be electrically balanced by an equivalent transport of a strong ion (Felle, 2005). Consequently, it is tempting to propose that coexcretion of \( \text{H}^+ \) and \( \text{NO}_3^- \) in vivo would allow the attenuation of stress-generated acidic drifts.

Possible Biological Relevance of NAXT-Mediated Root \( \text{NO}_3^- \) Efflux

Root \( \text{NO}_3^- \) efflux to the external medium is a component of net \( \text{NO}_3^- \) uptake. It may account for 80% of root \( \text{NO}_3^- \) influx in well-supplied plants (Kronzucker et al., 1999). In standard culture conditions, \text{naxt1}\#_mutants displayed no efflux phenotype and their growth or \( \text{NO}_3^- \) content was similar to those of wild-type plants. Thus, NAXT1 is not likely to play a role in plant N nutrition in standard culture conditions. Nevertheless, in this condition, \( \text{NO}_3^- \) efflux in vitro was inhibited using an antibody directed against the whole NAXT protein family. Furthermore, partial suppression of gene expression of all NAXT members in naxt-RNAi plants led to a significant modification of both root and shoot \( \text{NO}_3^- \) contents. Together, these data support the hypothesis that the activity of one or several NAXT members (other than NAXT1) is involved in \( \text{NO}_3^- \) transport in plants in standard culture conditions. Two NAXT members (Atg45680 and Atg45710) expressed in the root epidermis or cortex (Arabidopsis eFP Browser, http://www.arabidopsis.org/efp/cgi-bin/efpWeb.cgi) could also be involved in \( \text{NO}_3^- \) efflux to the external medium.

Nitrate efflux at the PM is also involved in xylem loading. Several anion channels have been described at the electrophysiological level in xylem parenchyma cells (reviewed in Roberts, 2006). A NAXT member (Atg45700) is strongly expressed in the root stele (Arabidopsis eFP Browser, http://www.arabidopsis.org/efp/cgi-bin/efpWeb.cgi), raising the possibility that transporters from the NAXT family are also involved in this process. Interestingly, relative to wild-type plants, the \( \text{NO}_3^- \) content of a \text{naxt-RNAi} line was significantly higher in roots and lower in shoots, suggesting that root-to-shoot \( \text{NO}_3^- \) translocation is altered in this mutant.

Large increases in \( \text{NO}_3^- \) efflux associated with \( \text{NO}_3^- \) excretion were reported in stresses such as defoliation (Macduff and Jacksson, 1992), mechanical stress (Aslam et al., 1996), transplant shock (Dehlon et al., 1995), and pathogen attacks (Garcia-Brugger et al., 2006). Here, we show that NAXT1 is responsible for the prolonged root \( \text{NO}_3^- \) excretion regime induced by acid load or medium acidification, leading to a decrease in root \( \text{NO}_3^- \) content. With up to 80 mM \( \text{NO}_3^- \) in well-fed plants (Radcliffe et al., 2005), the vacuolar pool accounts for the major part of the cell \( \text{NO}_3^- \) content (Devienne et al., 1994) and is likely remobilized to sustain \( \text{NO}_3^- \) cellular excretion. Consistently, NAXT1 is not expressed in root tip meristematic cells (Figure 7E) known to display very small vacuoles (Patel et al., 1990) but is expressed preferentially in mature cortical cells displaying large differentiated vacuoles.

Strong decreases in cell \( \text{NO}_3^- \) content due to prolonged \( \text{NO}_3^- \) excretion (Wendehenne et al., 2002) and early cytosol acidification (Mathieu et al., 1996; Roos et al., 1998) have been reported upon pathogenic attacks or application of elicitors of defense reactions. Additionally, acidification of plant medium has been shown to stimulate root \( \text{NO}_3^- \) efflux to the outer medium (Aslam et al., 1995). It should be noted that considerable variations in soil pH may occur within a region, depending on the type of soil and seasonal changes in soil moisture, temperature, microbial activity, and plant growth. Highly to extremely acidic soil solutions are not rare situations in natural environments (Soil Survey Staff, 1999). These data provide possible biological significance for the NAXT1-mediated \( \text{NO}_3^- \) excretion observed here upon root cell acidification.

In conclusion, a transporter endowed with \( \text{NO}_3^- \) excretion activity has been identified. NAXT1 mutants and transformants will likely help to clarify the implication and role of NAXT1 in stresses generating cytosolic pH drifts and/or \( \text{NO}_3^- \) excretion. More generally, characterization of members of the NAXT family is expected to lead to a better understanding of the enigmatic role of \( \text{NO}_3^- \) efflux in plants.
METHODS

Plant Cell Culture, Plant Growth Conditions, and Mutant Line Genotyping

Tobacco BY2 (Nicotiana tabacum cv Bright Yellow 2) cell suspensions (Nagata et al., 1992) were cultured in the dark at 26°C in Murashige and Skoog medium, pH 5.6, containing Murashige and Skoog salt (Murashige and Skoog, 1962), 1 mg/L thiamine-HCl, 0.2 mg/L 2,4-dichlorophenylacetic acid, 100 mg/L myo-inositol, 30 g/L sucrose, 200 mg/L KH2PO4, and 2 g/L MES. Cells were maintained by weekly dilution (1:40) into fresh medium.

Arabidopsis thaliana ecotype Columbia cell suspensions were cultured under continuous light at 24°C in Jouanneau and Pétáud-Lenoir medium as described (Axelos et al., 1992). They were subcultured with a 1:9 dilution factor every 7 d and used for membrane isolation at 7 days after transfer.

For GUS analyses of 7- and 14-d-old plants and to phenotype 14-d-old naxt-RNAI plants, Arabidopsis plants were grown in vitro in twofold-diluted Murashige and Skoog culture medium. For other analyses, Arabidopsis plants were grown for 6 weeks in hydroponic conditions as described (Lejay et al., 1999). Unless stated otherwise, the source of NO3- was 1 mM NH4NO3. Acid load treatment was performed by adding K-propionate (10 mM final concentration, pH 6.0) in the plant nutrient medium. Medium acidification treatment (pH 3) was performed by adding H2SO4 (5 N) in the nutrient medium, and the pH was controlled every hour.

HR 10/10 (Waters) column with discontinuous KCl gradients (25 or 100 mM steps). Protein fractions from tobacco PM were reconstituted into liposomes at a lipid:protein ratio of 30:1 (w/w) as described (Grouzis et al., 1997) to determine the NO3- efflux and permeability coefficient to NO3- using oxonol VI dye (see below). The PM from Arabidopsis suspension cells was stripped, and intrinsic proteins were solubilized by n-dodecyl β-d-maltoside and separated by GFC/IEC/SDS-PAGE as described (Szponarski et al., 2007). Protein separation by PAGE was performed according to Laemmli (1970). Gels stained with Coomassie Brilliant Blue R 250 (Bio-Rad) were scanned (GS-710 calibrated imaging densitometer; Bio-Rad) and analyzed using ImageMaster 1D Elite software (Amersham Biosciences). Candidate polypeptide bands were excised from the gel, digested with trypsin, and analyzed by matrix-assisted laser-desorption ionization time-of-flight MS as described (Szponarski et al., 2004).

Functional Assays

In vivo, root 15NO3- unidirectional efflux (Φco) and influx (Φoc) were measured according to Delhon et al. (1995) using a mass spectrometer (TraceMass; Europas Scientific) especially fitted with a channel calibrated for accurate measurement of 15N:14N ratio in the 80 to 100% range to determine Φco and with another classical channel for accurate measurement in the 0 to 20% range to determine Φoc. This setup made it possible to measure both Φco and Φoc on the same plants. The NO3- concentration (and the overall ion composition) in the labeling solution was the same as in the culture medium, except that a 99% 15NO3:14NO3 ratio was fixed at the beginning of the experiment. Then, Φco was determined from the amount of 15NO3 incorporated into the roots during a short period (5 min) and Φoc was determined from the dilution of 15NO3 in the labeling solution by 14NO3 originating from the roots during the same period.

In vitro, passive NO3- transport activity (Φoc, transport driven by Eoc) was measured on isolated PM vesicles polarized by one of two different means. In the first method, vesicles were polarized by activating PM H+-ATPase in ionic conditions (Grouzis et al., 1997) in which the initial H+ excretion rate (VH) of the pump is greatly restricted by Eoc. Addition of NO3- to PM vesicles competent for passive transport then depolarizes the vesicles, triggering an increase of VH attributable to a passive equivalent entry of NO3- (ΔVH). Eoc and ΔP were monitored with the oxonol VI and ACMA fluorescent dyes (Molecular Probes), respectively. In the second method, vesicles were polarized by imposing Li+ diffusion gradients in the presence of the ionophore ETH149, and Eoc was quantitatively monitored using oxonol VI (Poilquin et al., 1999).

Comparison of the slow dissipation kinetics of Eoc in the absence and presence of NO3- allowed us to determine passive NO3- flux (see Supplemental Figure 3 online). The two transport assays were performed as described (Grouzis et al., 1997; Pouliquin et al., 1999), except that they were scaled down to a microplate format (1420 multilabel counter, Wallac Victor2; Perkin-Elmer). Functional inhibition assays with antibodies were performed as described (Gaymard et al., 1993).

Antibodies and Protein Gel Blots

The Ab2Y2 antibodies were isolated from an antiserum obtained as described by Green and Manson (1998) after injection into a rabbit of the 17-kD PAGE protein band isolated from a fast protein liquid chromatography fraction (Amersham Pharmacia Biotech) that correlated with NAXT activity. The AbNAXT was obtained after co-precipitation of the three keyhole limpet hemocyanin–coupled synthetic peptides, P1, P2, and P3 (see sequences in Figure 2B), into rabbits and immunopurification of the resulting antiserum against the peptides (Eurogentec). Except for the AbNAXT IgGs (see above) and for commercial antibodies, all antibodies used were IgGs purified from rabbit antisera using the Melon Gel IgG spin purification kit (Pierce) according to the manufacturer’s instructions. Alkaline phosphatase–coupled anti-rabbit and anti-mouse antibodies were from Promega, and...
the anti-AcV5 monoclonal antibody was from eBioscience. Protein gel blot analysis was performed according to the ICN Biomedicals Aurora "n protocol, except that the duration of the washing steps was doubled and the alkaline phosphatase substrate CDP-Star (Roche) was used. Both primary and secondary antibodies were used at a 1:20,000 dilution. Chemiluminescent signals were acquired using films or a LAS-3000 CCD imager (Fuji).

**Sequence Alignments and Phylogenetic Analysis**

NAXT protein sequences were aligned using the MUSCLE algorithm (Edgar, 2004) included in the BiOpen 2.2 sequence analysis software (AbOxygen) for Macintosh. Protein sequences from members of the PTR family were extracted from the PlantsT database (http://plantst.genomics.purdue.edu/), and redundant sequences were removed. Alignment and tree data were produced using the Infobiogen website resources (http://babel.infobiogen.fr:1984/). Sequences were aligned using ClustalW 1.8 with the Gonnet series protein weight matrix. The resulting alignment is shown in Supplemental Figure 4 online. Phylogenetic tree data were produced using the neighbor-joining method (Saitou and Nei, 1987) with bootstrap analysis (1000 replicates). The graphical output of the unrooted tree was produced using the Infobiogen website resources (http://babel.infobiogen.fr:1984/). Sequences were aligned using ClustalW 1.8 included in the BiOpen 2.2 sequence analysis software (Edgar, 2004) included in the BiOpen 2.2 sequence analysis software (Edgar, 2004) included in the BiOpen 2.2 sequence analysis software.

**Molecular Cloning and Plant Transformation**

The NAXT1 ORF was amplified by RT-PCR on total RNAs extracted from Arabidopsis cells (ecotype Wassilewskija) using primers 611L and 611R (see above) and cloned into the Stul site of pCRI-Blunt (Invitrogen), leading to clone pC6100. The protein sequence deduced from the ORF DNA sequence was identical to that of the Columbia ortholog, except for a single physicochemically conservative R-to-K substitution at amino acid 128.

The NAXT- RNAi construct was obtained as follows. A 296-bp-long ORF cDNA sequence of NAXT1 (nucleotides 457 to 753) was chosen for the RNAi construct because it is highly conserved among other NAXT members (80 to 93% identity). This cDNA fragment was amplified by PCR from pC6100 (see above) using primers X611L (5'-CGCATACCTTCTGAGCTGATATTTAGCTT-3') and X611R (5'-CAGTGAGAGACATTTGCAGCTTGCTTATG-3'). The PCR fragment was digested with XhoI and KpnI and cloned into the silencing vector pHannibal (Wesley et al., 2001) digested with the same enzymes, leading to pH611Kx. To integrate another copy of this NAXT1 fragment but in the opposite orientation and on the other side of the PDK intron in the vector, the original PCR fragment was digested by XbaI/ClaI and cloned in pH611Kx digested with the same enzymes, resulting in construct pH611KX. The NAXT RNAi cassette containing the CaMV 35S promoter, the NAXT1 sequence in the positive orientation, the PDK intron, the NAXT1 sequence in the negative orientation, and the octopine synthase terminator was excised from pH611KX with NotI and cloned into the plant transformation vector pGreen 29. The resulting construct was used for Agrobacterium tumefaciens–mediated transformation of Arabidopsis plants. Among 10 independent homozygous NAXT-RNAi lines, one was selected (line R20) that displayed the lowest expression levels for all NAXT genes.

The PNAXT1:GUS construct was obtained as follows. The sequence of 563 bp upstream of the NAXT1 initiation codon that constitutes the entire intergenic region between the NAXT1 gene and the upstream gene (locus At3g45640) was amplified by PCR from Arabidopsis (Columbia) genomic DNA using the primers X-500L (5'-GGCGGCCTCTAGACATACCTTCTGAGCTTCCCTAG-3') and ProNR (5'-TGAAAACTAGCTGCTGCTAAAATACATGACAG-3'). The amplified fragment was digested by XbaI/NcoI and cloned into the plant transformation vector pCAMBIA 1305.1 (http://www.camibia.org/) digested with the same enzymes. This led to clone pCS611, harboring the GUS gene under the transcriptional control of the NAXT1 promoter sequence. Another fusion construct with a larger promoter sequence (starting 1029 bp upstream of the NAXT1 ATG, within the upstream At3g45640 gene locus) was obtained and displayed similar GUS patterns (data not shown).

The P35S:NAXT1-GFP construct was obtained as follows. The NAXT1 ORF was cloned by PCR from pC6100 using the primers B611gL (5'-CCATATGCTGAGCTGATATTTAGCTTATG-3') and B611gR (5'-CACTGCGGATCCGAGAGACATTTGCAGCTTGCTTATG-3'). The resulting fragment was digested with BamHI and cloned into the BamHI site of the p35S:GFP vector (Clontech). This led to clone pG611, in which the stop codon of NAXT1 was mutated to a Ser codon, leading to a C-terminal translational fusion of the NAXT1 ORF and the downstream GFP gene.

The naxt1-1 mutant complemented with NAXT1 was obtained as follows. The NAXT1 ORF was amplified by PCR from pC6100 using the primers X611L (5'-CGCATACCTTCTGAGCTGATATTTAGCTTATG-3') and X611R (5'-CACTGCGGATCCGAGAGACATTTGCAGCTTGCTTATG-3'). The PCR fragment was first cloned into pBSK [P70/rbs], a generous gift from D. Luu (Centre National de la Recherche Scientifique). This plasmid is a pBluescript SK− vector (Stratagene) in which the SacI, XbaI, and Xhol sites were first inactivated and in which a P70 promoter (CaMV 35S promoter with a duplicated enhancer)–rbs terminator cassette was inserted into the EcoRI/Clai sites after excision from pKYLX 71 (Scharld et al., 1987). This insertion reintroduced new unique XhoI, XbaI, and SacI sites between the promoter and terminator sequences. The NAXT1 ORF PCR fragment was cloned in the XbaI site, and constructs with inserts in the correct orientation with respect to the binary vector promoter and terminator sequences were selected. The P70-NAXT1-rbs cassette was then excised with SpeI/KpnI and subcloned into the binary vector pCAMBIA1300 digested with XbaI/KpnI. The resulting construct, pCA611, was used for Agrobacterium-mediated transformation of the naxt1-1 mutant.

The P70-NAXT1-AcV5 construct was obtained as follows. The NAXT1 ORF sequence was fused to the DNA of the AcV5 epitope (SWKADGWS) (Lawrence et al., 2003) by PCR amplification from pC6100 using the primers X611L and 611V5R2 (5'-CCAGCATCTGAGCTGATATTTAGCTTATG-3'). The resulting fragment was digested by XhoI/KpnI and cloned into the binary vector pCAMBIA1300 digested with XbaI/KpnI. The resulting construct, pCA611V, was used for Agrobacterium-mediated transformation of wild-type plants. All constructs were verified by sequencing. Arabidopsis plants were transformed using the floral dip immersion protocol described by Clough and Bent (1998).

**GFP Analyses**

Transient expression of the P35S-NAXT1-GFP construct was performed after transforming protoplasts of Arabidopsis cells with pG611 (see above) according to Leon et al. (2002). Protoplasts were observed at 48 and 72 h after transformation with a confocal laser microscope (Zeiss LSM510). GFP fluorescence was excited using the 480-nm line from an argon laser, and the fluorescence emitted was collected after passage through a 500- to 530-nm band pass filter. Image acquisition and treatments were performed using the LSM 5 Image Browser software (Zeiss).

**Gene Expression Analysis**

GUS analyses were performed according to Jefferson et al. (1987). RT-PCR experiments were performed as follows. Total RNAs were extracted from Arabidopsis roots using the RNeasy Plant Mini kit (Qiagen). Reverse transcription of 2.5 μg of total RNA was performed using Moloney

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murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. PCR (20 cycles, unless specified otherwise) was performed on 1 μL of first-strand cDNA using GoTaq DNA polymerase (Promega), using NAXT primer pairs whose sequences are given in Supplemental Table 1 online. The amplification specificity was verified by digesting the ~750-bp amplicon with a unique restriction enzyme (see Supplemental Table 1 online) that specifically cuts the amplified region in the cDNA sequence of the analyzed NAXT1 member but not the amplicons of other NAXT cDNAs. Specificity for PCR amplification of NAXT1 cDNA was also verified by sequencing the amplified product.

Control PCR amplification of EF1α cDNA was performed using the primers TEFA1F (5’-ATGGGTAAAGAAGTTTCATGAC-3’) and TEFA1R (5’-ACCAATCTTGATAGACATCTGAAG-3’).

Other Methods

Protein concentrations were estimated according to Bradford (1976). Nitrate content in root samples was measured according to Muños et al. (2004).

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource database (http://www.Arabidopsis.org) under accession numbers At3g45650 (NAXT1), At1g21120 (NRT1:1), At1g69850 (NRT1:2), At2g21670 (NRT1:3), At2g26690 (NRT1:4), At3g54140 (PTR1), At2g02040 (PTR2), and At5g46050 (PTR3).

Supplemental Data

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

**Supplemental Figure 1.** NAXT1 Gene Expression is Regulated Neither by Light Nor by Plant N Status.

**Supplemental Figure 2.** NAXT1 Gene Expression Is Abolished in naxt1 Mutant Lines.

**Supplemental Figure 3.** Determination of Net Passive Nitrate Fluxes from Dissipation of Diffusion Potentials Measured with the Oxonol VI Fluorescent Probe.

**Supplemental Figure 4.** Alignment of Protein Sequences of Members of the NRT1/PTR Family Used to Generate the Tree Presented on Figure 2A.

**Supplemental Table 1.** Primer Sequences Used for RT-PCR Analysis of NAXT Members.

**Supplemental Table Set 1.** Text File of ClustalW Protein Sequence Alignments of Members of the NRT1/PTR Family Used to Generate the Tree Presented in Figure 2A.

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# Nitrate Efflux at the Root Plasma Membrane: Identification of an Arabidopsis Excretion Transporter

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