**Arabidopsis NIP2;1, a Major Intrinsic Protein Transporter of Lactic Acid Induced by Anoxic Stress**

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Nodulin 26 intrinsic proteins (NIPs) are plant-specific, highly conserved water and solute transport proteins with structural and functional homology to soybean nodulin 26. *Arabidopsis thaliana* contains nine NIP genes. In this study, it is shown that one of these, *AtNIP2;1*, is exquisitely sensitive to waterlogging and anoxic stress. Based on quantitative PCR and promoter::GUS experiments, *AtNIP2;1* is expressed at a low basal level in the root tips and the vascular bundle of differentiated roots. Transcript levels are elevated acutely and rapidly upon water logging of root or leaf tissues, increasing 70-fold in roots within the 1st h of submersion. After this large increase, mRNA levels decline to steady state levels that remain over 10-fold higher by 6 h post-submersion. An even greater induction of *AtNIP2;1* expression was observed upon anoxia challenge of *Arabidopsis* seedlings, with a 300-fold increase in *AtNIP2;1* transcript observed by 2 h after the initiation of oxygen deprivation. Functional analysis of *AtNIP2;1* expressed in Xenopus oocytes shows that the protein differs from soybean nodulin 26, showing minimal water and glycerol transport. Instead, *AtNIP2;1* displays transport of lactic acid, with a preference for the protonated acidic form of this weak acid. Overall, the data suggest that *AtNIP2;1* is an anaerobic-induced gene that encodes a lactic acid transporter and may play a role in adaptation to lactic fermentation under anaerobic stress.

Major intrinsic proteins (MIPs) are an ancient integral membrane channel protein family of water and uncharged solute transporters that have been found in nearly all living organisms. MIPs are especially prevalent and diverse in higher plants and have been classified into four monophyletic groups as follows: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins, the nodulin 26-like intrinsic proteins (NIPs), and the small basic intrinsic proteins (1).

NIPs are named for soybean nodulin 26 (Nod26) (2), which is the major protein component of the symbiosome membrane from nitrogen-fixing soybean root nodules (3, 4). Functional analyses indicate that Nod26 is an aquaglyceroporin with a low intrinsic water permeability and the ability to transport uncharged metabolites such as glycerol (4, 5) and has also been implicated in ammonia transport (6). It has become clear that NIPs represent a large, diverse family of aquaglyceroporins, with multiple members found in every sequenced higher plant genome (1, 7, 8). For example, among the 35 MIP genes in *Arabidopsis thaliana* (1), there are nine members of the NIP subfamily. Based on molecular modeling of the pore selectivity sequences, these nine NIPs are subdivided into the following two groups: NIP subgroup I proteins are encoded by *NIP1;1, NIP1;2, NIP2;1, NIP3;1, NIP4;1, and NIP4;2*, and NIP subgroup II proteins are encoded by *NIP5;1, NIP6;1, and NIP7;1* (9).

Analysis of NIP subgroup I proteins shows that they are more similar to soybean nodulin 26 in structure and function, with several showing aquaglyceroporin activities (10–14 and reviewed in Ref. 15). NIP subgroup II proteins on the other hand form glyceroporins with an exceedingly low water permeability (16, 17), and also transport other uncharged substrates, including urea (17, 18) as well as metalloid nutrients such as boron (19) and silicon (20). Overall, the observations suggest that NIPs are likely involved in transport functions other than water flux.

In this study we show that the NIP subgroup I protein, *Arabidopsis* NIP2;1, is a lactic acid transporter with an unusually low water permeability that is expressed predominantly in the vascular tissues of roots. Furthermore, we show that *AtNIP2;1* expression is exquisitely sensitive to flooding stress and oxygen deprivation. This observation, along with the lactic acid transport selectivity of the protein, suggests a role in metabolic adaptation to oxygen deficit.

**EXPERIMENTAL PROCEDURES**

**Plant Growth and Transformation—** *A. thaliana* ecotype Columbia 0 seeds were vernalized on ½-strength Murashige and Skoog (MS) agar medium containing 1.5% (w/v) sucrose for 2 days at 4 °C, and were then grown under a long day (LD) cycle of 16 h light/8 h dark at 22 °C. Twelve-day-old seedlings were transplanted to Pro-Mix soil (Premier Horticulture Inc., Dorval, Quebec, Canada) and were grown under cool white fluorescent lights (76 – 100 μmol m⁻² s⁻¹) at 22 °C under the LD cycle.

For flooding stress experiments, seeds were germinated and grown vertically on the grid A line of square Petri dish plates (gridded 100 × 100 × 15-mm plates; Fisher) containing ½-strength MS agar medium.Flooding stress was administered by submerging the root region of 2-week-old seedlings to the grid B line, and root samples were harvested at intervals over 24 h.
Nodulin-like Transporter of Lactic Acid

For anoxia stress experiments, seeds were germinated on sterile filter paper and were grown on ½-strength MS agar medium under the LD cycle. At 10 days, seedlings were placed into an anaerobic jar, and anoxia was achieved using a BBL GasPak 100 System (BD Biosciences). Seedlings were harvested at various intervals, immediately frozen in liquid nitrogen, and then stored at −80 °C until RNA isolation.

Plant transformation was carried out using the floral dip method (21). Plant inflorescences were submerged into mid-logarithmic cultures (A660 = 0.8) of Agrobacterium tumefaciens strain GV3101 (22) in 5% (w/v) sucrose and 0.05% (v/v) Silwet-L77 (Lehle Seeds, Round Rock, TX) for 1 min, and plants were kept overnight in a growth chamber set to LD conditions. Plants were washed 3–5 times with water and grown under LD conditions until seed set. Germination of seed and selection of transformants were done on ½-strength MS agar containing 50 μg ml−1 hygromycin.

Molecular Cloning Techniques—For the AtNIP2;1 promoter:GUS reporter construct, a DNA fragment corresponding to 1098 bp of the AtNIP2;1 gene upstream of the transcriptional start site was amplified by PCR using gene-specific primers with HindIII and PstI sites introduced for cloning (supplemental Table S1). The PCR-amplified AtNIP2;1 promoter fragment (1098 bp) was digested with HindIII and PstI and was cloned into the HindIII and PstI sites of pCAMBIA1391Z (23) upstream of the promoterless GUS reporter gene.

Xenopus oocyte expression constructs of the open reading frame of the AtNIP2;1 cDNA were generated from Arabidopsis root total RNA (6 weeks Arabidopsis) by reverse transcription-PCR amplification using gene-specific primers with BglII sites (supplemental Table S1). The amplified cDNA fragment was cloned into the BglII restriction site of a modified Xenopus expression plasmid pXβG-ev1 containing a sequence to introduce an in-frame N-terminal fusion of the FLAG epitope (MDYKDDDDK) as described in Ref. 17. A FLAG tag fusion of soybean nodulin 26 was generated in the same vector. Capped cRNA was generated by in vitro transcription of Xbal-linearized pXβG-ev1 constructs by using the mMESAGeMACHINE T3 kit (Ambion, Austin) as described previously (17, 24).

For subcellular localization experiments, a cDNA encoding the full-length AtNIP2;1 open reading frame was amplified using gene-specific primers (supplemental Table S1) with NcoI sites introduced for cloning into the expression vector pBS-3SS-YFP (25) downstream of cauliflower mosaic virus 35S promoter in-frame with a C-terminal yellow fluorescence protein (YFP) tag.

For the preparation of transgenic Arabidopsis expressing the AtNIP2;1::YFP fusion, a cassette consisting of CaMV 35S promoter-AtNIP2;1::YFP fusion was cloned into the BamHI site of the pBIN19 plant binary vector (26). All plasmids were transformed into Escherichia coli DH5α. Sequences were verified by automated DNA sequencing using a model 373 DNA sequencer (Applied Biosystems) at the University of Tennessee Molecular Biology Research Facility (Knoxville, TN).

Total RNA Isolation and Quantitative Real Time PCR (Q-PCR)—Total RNA was isolated from tissue samples (200 mg) by using the Plant RNA Purification Reagent (Invitrogen). Genomic DNA was removed by RNase-free DNase I treatment using the DNA-free™ kit (Ambion, Austin) according to the manufacturer’s instructions. Total RNA (2 µg) was reverse-transcribed into cDNA in a 20-µl reaction (100 ng of total RNA/µl) with the SuperScript™ first-strand synthesis system for reverse transcription-PCR (Invitrogen). The quality of first strand cDNA samples was monitored by PCR analysis of the Arabidopsis Actin2 reference gene. Q-PCR analysis was done on an ABI Prism 7000 sequence detection system, and analysis was performed with the ABI Prism 7000 SDS software (Applied Biosystems). Gene-specific and internal control primers are described in supplemental Table S2. The Arabidopsis UBQ10 gene was used as an internal reference for standardization as described previously (27). cDNA proportional to 10–100 ng of starting total RNA was combined with 200 nm of each primer and 12.5 µl of 2XABsolute SYBR Green ROX dUTP mix (ABgene, Rochester, NY) in a final volume of 25 µl. Q-PCRs were performed using the following parameters: 1 cycle of 5 min at 50 °C, 1 cycle of 5 min at 95 °C, and 45 cycles of 30 s at 95 °C, 45 s at 45 °C, and 45 s at 72 °C in a 96-well optical PCR plate (ABgene). Quantitation of AtNIP2;1 expression was calculated using the comparative threshold cycle (Ct) method as described previously (28). ∆ΔCt was calculated using Equation 1,

\[
\Delta\Delta Ct = \Delta Ct(\text{target}) - \Delta Ct(\text{reference})
\]

where \(\Delta Ct(\text{target})\) is the Ct value of gene of interest, and \(\Delta Ct(\text{reference})\) is Ct value of the Arabidopsis UBQ10. ∆ΔCt values were calculated using Equation 2,

\[
\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{calibrator})
\]

where \(\Delta Ct(\text{sample})\) represents the expression value of the gene of interest calculated using Equation 1, and \(\Delta Ct(\text{calibrator})\) is the expression value of the sample to which other samples in the data set are normalized. Each \(\Delta Ct(\text{calibrator})\) of individual Q-PCR experiments are indicated in each figure legend. The relative expression value was obtained from ∆ΔCt values by using Equation 3,

\[
\text{relative expression} = 2^{-\Delta\Delta Ct}
\]
initial oocyte volume at time 0, calculated as described previously (17, 24).

Direct glycerol and urea permeability measurements of Xenopus oocytes were performed by radioisotopic uptake assay as described in Ref. 17. Lactic acid transport experiments in oocytes were done by a similar approach except that 14C-labeled lactic acid was used. The assay buffer consisted of a modified Ringer’s solution containing 20 mM lactic acid (12 μCi/ml 14C-labeled lactic acid (Sigma)) in a base buffer of 75 mM NaCl, 2 mM KCl, 5 mM MgCl2, 5 mM Tris succinate, 0.6 mM CaCl2 (200 mosm/kg). Assay incubations were done at 22 °C for 10 min, and oocytes were washed twice with 6 ml of ice-cold Ringer’s solution without isotope. Sensitivity to mercurials was determined by preincubating oocytes in Ringer’s solution containing 1 mM HgCl2 for 10 min prior to assay, essentially as described previously (4). After isotopic uptake assays, oocytes were lysed with 300 μl of 10% (w/v) SDS, and scintillation counting was done in 10 ml of Scintsafe (Fisher) by using a Beckman LS6500 multipurpose scintillation counter (Beckman Instruments, Fullerston, CA).

Histochemical and Immunochemical Methods—GUS staining was done on 2-week-old Arabidopsis as described in Ref. 29 with slight modifications. Tissues were incubated for 8–16 h at 37 °C in 0.1 M potassium phosphate, pH 7.0, 0.1% (w/v) Triton X-100, 0.4 mM K3[Fe(CN)6], 0.4 mM K4[Fe(CN)6], and 0.9 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase (Rose Scientific, Ltd, Edmonton, Alberta, Canada). Seedlings were cleared with 70% (v/v) ethanol at room temperature and were mounted in 50% (w/v) glycerol. Stained tissues were observed and imaged using a Nikon ECLIPSE E600 microscope equipped with MicroPublisher 3.3 cooled and QCapture 2.60 software (QImaging Corp., Burnaby, British Columbia, Canada).

Transient expression of AtNIP2;1-YFP in mesophyll protoplasts prepared from 3-week old Arabidopsis Col_0 wild type plants was done by the protocol described in Ref. 30. Protoplasts were resuspended (2 × 106 protoplasts/ml) in 0.4 M mannitol, 15 mM MgCl2, 4 mM MES, pH 5.7, and were transformed with 10 μg of pBS-35S-YFP containing the AtNIP2;1-YFP construct by the procedure described previously (30). Protoplasts were cultured at room temperature for 18 h in 1 ml of 154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES, pH 5.7. Subcellular localization of AtNIP2;1-YFP was observed using a Leica DMRE laser-scanning confocal microscope with filter settings of 507–532 nm for YFP and 588–716 nm for the chloroplast signal collection at the University of Tennessee Analytical Microscopy Facility (Knoxville, TN).

Stable transgenic Arabidopsis lines overexpressing AtNIP2;1::YFP C-terminal fusion were generated as described under “Plant Growth and Transformation.” AtNIP2;1::YFP was visualized in primary root tissues from 7-day-old T1 generation of AtNIP2;1::YFP expression lines using an Axiovert 200 M microscope (Zeiss) equipped with YFP fluorescence filter setting (Chroma, filter set 52017) of 500–530 nm. Images were captured with a digital camera (Hamamatsu Orca-ER) controlled by the Openlab software (Improvision). Western blots for FLAG-tagged proteins in Xenopus oocytes were done as in Ref. 17.

**RESULTS**

AtNIP2;1 Is Expressed in the Vascular Tissue of Arabidopsis Roots and Is Induced by Anoxia Stress—Consistent with previous observations of microarray (31) and promoter-reporter fusion data (32), Q-PCR analysis of total RNA from various organs of 2-week old Arabidopsis shows that AtNIP2;1 transcript is predominantly expressed in roots compared with other organs (Fig. 1A). GUS expression analysis supports the findings of Q-PCR and shows that the AtNIP2;1 promoter drives the expression of GUS in root tissues (Fig. 1, B and C). Expression predominantly occurs within the root cap and within the vascular cylinder of mature cells within the zone of cell specialization of the primary root, but it appears to be lacking in the zone of cell division and elongation (Fig. 1, B and C). Additionally, staining is absent in emerging, elongating lateral roots (Fig. 1B).

To determine the subcellular localization of AtNIP2;1, C-terminal YFP fusion constructs were generated and were used to transiently transform Arabidopsis mesophyll protoplasts as well as produce stably transformed transgenic Arabidopsis plants (Fig. 2). Transient expression of AtNIP2;1-YFP results in a uniform expression around the cell periphery of protoplasts with a localization distinct from the cytosolic compartment visualized with endogenous fluorescence from chloroplasts (Fig. 2, A–C), consistent with plasma membrane localization. In addition, CaMV 35S-driven expression of AtNIP2;1::YFP in transgenic Arabidopsis roots shows a similar pattern of fluorescence (Fig. 2D), again consistent with localization in the plasma membrane.
conditions tested, AtNIP2;1 expression showed extreme sensitivity to flooding (Fig. 3). Water logging of the roots of 2-week-old young seedlings results in a 70-fold increase in AtNIP2;1 transcript levels within the 1st h after submersion as assayed by Q-PCR (Fig. 3A). Expression decreased by 6 h post-flooding but still remained between 10- and 20-fold higher than the control transcript levels (Fig. 3A). This expression profile is also apparent in the GUS staining pattern of AtNIP2;1 promoter::GUS transgenic Arabidopsis plants exposed to flooding stress (Fig. 3B). After submersion, GUS expression rapidly increased and after 1 h peaked with high expression in the vascular cylinder as well as in cortical cells and lateral roots (Fig. 3B). GUS expression declined after this point, reaching a stable but elevated level at 6 h (Fig. 3B). Water logging of roots results in severe oxygen deficit because of the low diffusion coefficient of oxygen in water (33).

To test whether elevation of AtNIP2;1 expression is part of the response of the plant to oxygen deficit, 10-day-old Arabidopsis seedlings were subjected to anoxia and AtNIP2;1 expression, along with that of two established anaerobic polypeptide transcripts (Pdc1 and Adh1 encoding pyruvate decarboxylase 1 and alcohol dehydrogenase 1, respectively (34, 35)), and were analyzed by Q-PCR (Fig. 4). At 30 min after the onset of anoxia, AtNIP2;1 exhibits an increase in expression that parallels that of Pdc1 (Fig. 4A) and Adh1 (Fig. 4B), and by 2 h the expression of AtNIP2;1 is increased 300-fold compared with control levels (Fig. 4B).

**Other Arabidopsis NIP Genes Are Not Affected by Anaerobic Stress**—As pointed out previously, AtNIP2;1 is a member of a multigene subfamily of Arabidopsis NIPs (1, 15). To determine whether this sensitivity to waterlogging/oxygen deprivation is a common response among the NIP subfamily, Q-PCR was performed on flooded and anoxia-stressed seedlings using transcript-specific probes for all members of the NIP subgroup I (AtNIP1;1, AtNIP1;2, AtNIP2;1, AtNIP3;1, AtNIP4;1, and AtNIP4;2). As shown in Fig. 5, a low but detectable signal was observed for all NIPs in 10-day-old Arabidopsis seedlings, except AtNIP4;1, which is expressed at an exceedingly low level based on microarray and Q-PCR data (31). Although AtNIP1;1 showed a slight increase in expression in response to water logging, all other NIP transcripts showed little or no change in response to flooding or anoxia stress compared with AtNIP2;1 (Fig. 5A). This argues that AtNIP2;1 is selectively regulated in response to oxygen deprivation.

**Analysis of the Water and Solute Permeability of AtNIP2;1**—To determine its water and solute transport properties, AtNIP2;1 was expressed as an N-terminal FLAG-tagged fusion in Xenopus oocytes. AtNIP2;1 is a member of NIP subgroup 1 (9), and for the sake of comparison to this group of proteins, its
transport properties were compared with the well studied prototypical NIP1 protein, soybean nodulin 26, by using the general approach of Wallace and Roberts (17).

Injection of AtNIP2;1 cRNA results in expression of the protein in oocytes at approximately the same level as soybean nodulin 26 (Fig. 6) but showed a lower ability to transport water. AtNIP2;1-expressing oocytes showed only a modest 2-fold increase in their osmotic water permeability ($P_f = 0.99 \times 10^{-4}$ cm/s, pH 7.6), compared with control oocytes (0.46 $\times 10^{-4}$ cm/s), and was significantly lower than that observed with control oocytes expressing soybean nodulin 26 (1.93 $\times 10^{-4}$ cm/s, pH 7.6) (Fig. 6). Water flux through AtNIP2;1 and nodulin 26 were both inhibited by 1 mM Hg$^{2+}$ (Fig. 6B), consistent with previous observations suggesting protein facilitated water flow (4). Interestingly, nodulin 26 showed an enhanced $P_f$ in response to lower pH ($4.6 \times 10^{-4}$ cm/s, pH 5; Fig. 6A), consistent with previous observations of pH-dependent gating stimulating the transport of some aquaporins (36). The water permeability of AtNIP2;1 remained low throughout the pH range, although a slight elevation of $P_f$ is observed at pH 4 (Fig. 6A).

As discussed previously, given the low intrinsic water permeability of the NIP family and their multifunctional transport activities, they may play a cellular role in the transport of alternative uncharged metabolites (15). Given the observation that AtNIP2;1 expression is elevated in response to flooding stress, consideration of a transport activity supporting adaptation to this stress was considered. Flooding stress in plants results in oxygen deficit, which induces a rapid metabolic shift from aerobic respiration to lactic acid fermentation (37). As part of the adaptation to this altered metabolic flux, and to avoid cytosolic acidification, several plant species acquire the ability to transport lactate/lactic acid out of the cytosol (38). An examination of protonated lactic acid ($M_f = 90.1$ and van der Waals volume = 48.0 cm$^3$/mol) revealed similarities in solute size and dimension to other NIP transport substrates (for example, glycerol with $M_f = 92.1$ and van der Waals volume = 514 cm$^3$/mol). To test the hypothesis that AtNIP2;1 might be involved in transport activities associated with anaerobic adaptation, we analyzed the transport behavior of the protein upon expression in Xenopus oocytes.

Oocytes expressing AtNIP2;1 show an enhanced rate of uptake of $[^{14}\text{C}]$lactic acid from the bath solution, which is dependent on pH (Fig. 7). Furthermore, the pH dependence of the transport rate of $[^{14}\text{C}]$lactic acid parallels the calculated concentration of protonated lactic acid (Fig. 7B), suggesting that the acid form is the substrate for transport.

Uninjected oocytes also show an enhanced uptake of $[^{14}\text{C}]$lactate at lower pH, albeit at a much lower rate (Fig. 7A). However, as shown in Fig. 8, transport of lactic acid in AtNIP2;1-expressing oocytes shows the hallmark of facilitated, protein-mediated transport. First, similar to findings with water and glycerol transport through other NIPs, AtNIP2;1 lactic acid transport is inhibited by mercurial compounds (Fig. 8A). Furthermore, analysis of the activation energy of transport in uninjected and AtNIP2;1-expressing oocytes was analyzed by Arrhenius plot (Fig. 8B). Calculation of the Arrhenius activation energy shows that AtNIP2;1 lowers the activation energy of lactic acid uptake ($E_a = 4.02$ kcal/mol) compared with unin-
jected oocytes \( (E_a = 15.1 \text{ kcal/mol}) \), consistent with facilitated transport of water and solutes through aquaporin/glyceroporin channels (39). Finally, transport through AtNIP2;1 shows saturable kinetics (Fig. 7C, apparent \( K_{0.5} \) = 34.7 mM (S.E. = 3.5)) in contrast to lactic acid transport in control uninjected oocytes, which show a slow and unsaturable rate (data not shown). Overall, the data strongly suggest transport of lactic acid through the AtNIP2;1 protein.

Interestingly, in contrast to soybean nodulin 26, AtNIP2;1-expressing oocytes showed minimal transport of glycerol regardless of pH (Fig. 9B). Other NIP transport substrates such as urea (17) and boric acid (19) are also not fluxed by AtNIP2;1 (Fig. 7C and Table 1). Conversely, nodulin 26-expressing oocytes were indistinguishable from negative control oocytes with respect to \(^{14}\text{C}\) lactic acid uptake (Fig. 9A), suggesting a distinct transport selectivity for these two NIPs.

As a final note, the ability of AtNIP2;1 to transport ethanol, a product of ethanolic fermentation product during oxygen stress (40), was evaluated. As shown in Table 1, the permeability of AtNIP2;1 oocytes to ethanol is essentially not different from that of control oocytes. Overall, the data support the contention that AtNIP2;1 is a selective transporter of the protonated form of lactic acid.

**DISCUSSION**

Expression analyses in model organisms such as Arabidopsis, rice, and maize suggest that NIP genes are in general expressed at a much lower level compared with most other plant MIPs (1, 7, 8). In addition, they are often expressed in specialized cells and organs suggesting that NIP transport activities may be prevalent in a more defined set of cells in the plant (15). The present experiments with AtNIP2;1 support this contention, with low basal levels of expression apparent predominantly in the vascular tissues of mature roots as well as in the root tip. Moreover, AtNIP2;1 is extremely sensitive to oxygen deprivation, showing a rapid and large increase in transcript levels in response to water logging or anoxia. Functional analyses of AtNIP2;1 show that it selectively transports protonated lactic acid, suggesting a role for this activity in adaptation of tissues to oxygen deficit.

Oxygen deficit resulting from stress such as flooding or water logging leads to severe depression of respiration resulting in reduced adenylate energy charge and the accumulation of toxic metabolites and cytosol acidification (38). Plants adapt to these conditions by employing several short and long term strategies as follows: 1) increases in glycolytic flux to provide ATP (the Pasteur Effect); 2) elevation of fermentation metabolism to regenerate NAD\(^+\) for glycolysis; and 3) ultimately morphological developmental changes (e.g. aerenchyma, adventitious root formation, and root and stem elongation) to elevate O\(_2\) levels in water-logged roots (37). The expression and translation of most genes and mRNAs are generally suppressed under hypoxic conditions because of the need to conserve energy. However, a set of genes encoding “anaerobic polypeptides” (40) are induced, which include glycolytic and fermentation enzymes, as well as various signal transduction proteins, transcription factors, and other genes involved in the adaptation response to anaerobiosis (41, 42). The results from
this study suggest that AtNIP2;1 is an anaerobic polypeptide in Arabidopsis.

An interesting parallel is also observed between patterns of AtNIP2;1 expression in plants grown under unstressed “normoxic” conditions and previous observations of the oxygen content of various tissues. For example, root tissues that show high expression of AtNIP2;1 are also the tissues that have low oxygen tension even under growth in sufficient O2. For example, the stele of mature root generally exhibits the metabolic symptoms of anoxia, likely due to poor radial diffusion of O2 (43, 44). Similarly, the root tip also exhibits a low O2 tension (43) presumably due to high metabolic rates and O2 consumption. Overall, these observations support the results of flooding and anoxia treatments and suggest that low oxygen concentrations is the critical factor inducing AtNIP2;1 expression.

With respect to transport function, AtNIP2;1-injected oocytes show an extremely low osmotic water permeability. Consistent with this finding, previous light scattering work

FIGURE 7. Lactic acid transport by AtNIP2;1 oocytes. AtNIP2;1F-expressing oocytes were tested for the uptake of lactic acid by incubation in bath solutions containing modified Frog Ringer’s solution with 14C-labeled lactic acid (20 min) as described under “Experimental Procedures.” A, 14C-labeled lactic acid transport rate of control or AtNIP2;1F-expressing oocytes as a function of the media pH. Error bars show S.E. (n = 3). B, lactic acid uptake rate of AtNIP2;1F-expressing oocytes as a function of pH. [14C]Lactic acid uptake was performed as in A with the exception that the media solution was buffered to the pH value indicated. The uptake by AtNIP2;1F oocytes was corrected by subtracting the basal lactic acid uptake by water-injected control oocytes. The solid line shows the concentration of the protonated lactic acid as a function of pH based on the Henderson-Hasselbalch equation. Error bars show S.E. (n = 3). C, selectivity of AtNIP2;1 for known NIP substrates is shown. Oocytes were assayed in 20 mM of the indicated radiolabeled substrate, and the uptake by AtNIP2;1F oocytes was corrected by subtracting the basal uptake by water-injected control oocytes. The error bars show the S.E. (n = 9 for lactic acid, n = 10 for urea, and n = 3 for glycerol).

FIGURE 8. Lactic acid transport by AtNIP2;1 shows the hallmarks of facilitated transport. A, effects of Hg2+ on the [14C]lactic acid uptake by AtNIP2;1F. Oocytes were preincubated with 1 mM HgCl2 for 10 min prior to assay of lactic acid uptake. Error bars show S.E. (n = 3). B, Arrhenius plot of lactic acid transport through control and AtNIP2;1 oocytes (n = 6). C, concentration dependence of AtNIP2;1 transport of lactic acid. Lactic acid uptake by AtNIP2;1F oocytes was done at the indicated concentrations, and uptake rates were corrected by subtracting the basal uptake by water-injected control oocytes. Error bars show S.E. (n = 3).
function of lactic acid transport emerges when one considers the metabolic changes associated with the onset of oxygen deprivation in roots.

Among the metabolic responses of plant roots to anaerobic stress is the rapid and transient induction of lactic acid fermentation followed by a switch to a sustained ethanolic fermentation (the Davies-Roberts hypothesis (46, 47)). This shift to the non-acid-producing ethanolic fermentation pathway is proposed to prevent over-acidification of the cytosolic compartment by excess production of lactic acid (47, 48). Consistent with this observation, lactic acid efflux is observed in hypoxia-challenged roots of certain plant species (49–51). Moreover, this induction of lactic acid secretion is correlated with an increased ability to sustain metabolic activity (49) and presumably mitigates the cytosolic acid load during flooding-induced fermentation (38).

To decrease cytosolic acidity from lactic acid fermentation, the transport of a proton, either as lactic acid or co-transport of \( \text{H}^+ \) and lactate, is required. In animal cells engaged in lactic acid fermentation, this role is performed by a proton-coupled monocarboxylic acid transporter that is induced under hypoxic conditions (52, 53). The mechanism for lactic acid efflux in oxygen-deprived plant roots is less clear. The transport properties of AtNIP2;1 suggest that it has the potential properties needed for a lactic acid efflux channel. For example, the pH profile of transport strongly suggests that AtNIP2;1 transports only the uncharged protonated form of lactic acid. This is consistent with the general properties of MIPs as transporters of uncharged metabolites and are resistant to permeation by charged species (39). Thus, the efflux of lactate would only occur in conjunction with a proton, lowering the acidity of the cytosol in the process. In addition, the rapid time course of expression is consistent with the rapid onset of lactic acid fermentation and accompanying lactic acid release from hypoxic roots, which is detectable within the 1st h after oxygen deprivation (49).

One interesting consideration in regard to its potential transport function is the subcellular localization of AtNIP2;1. In previous work using C-terminal AtNIP2;1-green fluorescence protein fusions and transient expression analysis in Arabidopsis suspension cell cultures, Mizutani et al. (32) showed predominant localization to an internal compartment consistent with the endoplasmic reticulum. In contrast, in the present work using both transient expression in mesophyll protoplasts and transgenic Arabidopsis roots, an AtNIP2;1-yellow fluorescence protein fusion appears to be localized to the surface of the cell, presumably the plasma membrane. However, similar to Mizutani et al. (32), observations of fluorescence in internal membrane compartments was sometimes observed with mesophyll protoplasts (data not shown). It is noteworthy that some aquaporins, such as AQP2 in mammalian cells, can be observed both on internal membrane vesicles as well as the plasma membrane, with this localization being subject to regulation (54). Further analysis of the localization of native AtNIP2;1 under conditions of normoxia and anoxia is necessary to resolve this apparent discrepancy in localization.

As a final note, the transport properties of AtNIP2;1 are noteworthy from a structural and functional perspective of

TABLE 1

| Solute      | Transport rate \(^{a}\) | Transport rate \(^{b}\) |
|-------------|-------------------------|------------------------|
|             | Control                 | AtNIP2;1               |
| Boric acid  | (S.E. = 0.019, \( n = 8 \)) (S.E. = 0.014, \( n = 8 \)) | 0.123                  |
| Ethanol     | 0.195                   | 0.200                  |
| Lactic acid | (S.E. = 0.027, \( n = 7 \)) (S.E. = 0.029, \( n = 9 \)) | 0.033                  |

\(^{a}\) Assays were performed in isoosmotic modified Ringer’s solution with 100 mM of the indicated solute as described under “Experimental Procedures.” Upon immersion into the indicated solution, the uptake of solute was monitored by the rate of swelling as water follows the solute into the oocyte.

\(^{b}\) The transport rate was determined by measuring the rate of oocyte swelling upon transfer from Ringer’s solution into the modified Ringer’s solution with the indicated substrate. The rates represent the \((dV/V)_o\) as \( \times 10^3 \).

with microsomes from AtNIP2;1-transformed yeast shows only modest increases (50%) in water permeability (32). These observations, along with the general finding that aquaporin activities in roots are suppressed by flooding stress (45), suggest that AtNIP2;1 likely does not function as an aquaporin in vivo. In addition, unlike soybean nodulin 26 and other NIP aquaglyceroporins (5, 11–14), AtNIP2;1 shows low permeability to glycerol. The surprising finding is that the protein mediates the flux of lactic acid. A possible

FIGURE 9. Comparison of the glycerol and lactic acid uptake by AtNIP2;1 and soybean nodulin 26. A, \(^{14}\)C-labeled lactic acid uptake by oocytes expressing AtNIP2;1F or Nod26F or control oocytes injected with RNase-free water was done as described in Fig. 7. B, \(^3\)H-labeled glycerol uptake by oocytes expressing AtNIP2;1F or Nod26F or control oocytes injected with RNase-free water was done as described under “Experimental Procedures.” Standard assays were done at pH 7.6; however, the uptake by AtNIP2;1 was also performed at pH 4.0. The error bars show S.E. (\( n = 3 \)).
the NIP transport family. As pointed out previously, the pore properties and multifunctional transport signature of this subfamily of plant MIPs are unique (15). Based on modeling, there are two general pore subfamilies of NIP, NIP I and NIP II (9). NIP I proteins are typified by soybean nodulin 26 and form aquaglyceroporins that transport glycerol as well as water (4). In contrast, NIP II proteins, which differ principally at one residue within the aromatic/arginine selectivity filter, show little water permeability (17, 19) but do transport a variety of uncharged solutes, including glycerol and urea (17), as well as metalloid compounds, including boron and silicon (19, 20).

With respect to pore determinant sequences, AtNIP2;1 resembles the nodulin 26-like NIP I pore group (9), showing the conserved aromatic/arginine selectivity sequence of this group. Nevertheless, the results of the present study show that AtNIP2;1 is clearly distinct from nodulin 26 and other NIP I proteins, such as AtNIP1;1 and -1;2 (10, 11), not only in its ability to transport lactic acid instead of glycerol but also in its unusually low permeability to water. Modeling results using existing crystal structure templates do not provide any apparent leads for this distinction. In this regard, it is important to realize that although the MIP family in general consists of a conserved “hourglass” fold and topology (39), each MIP has unique regulatory and transport properties. For example, the recent structural determination of SoPIP2;1 from spinach reveals the importance of cytosolic loop and terminal regions in gating the transport through PIP aquaporins (55), and structures of mammalian aquaporins such as AQP0, which have low water permeability reveal other selectivity constrictions besides the classical Asn-Pro-Ala and aromatic/arginine pore selectivity regions (56). Further structural analyses of AtNIP2;1 are needed to reveal the molecular basis for its distinct transport selectivity relative to the soybean nodulin 26 archetype. In addition, because AtNIP2;1, like nodulin 26 and certain other NIP I proteins, contains a conserved phosphorylation site within its C-terminal domain (15), it will be of interest to determine whether phosphorylation plays a role in the regulation of its activity in planta.

Acknowledgments—We thank Dr. Albrecht von Arnim, University of Tennessee, for helpful comments during the course of this work. We also acknowledge Dr. Andreas Nebenfuehr, University of Tennessee, for assistance with the subcellular localization experiments of YFP protein fusions.

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