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NtPDR3, an iron-deficiency inducible ABC transporter in *Nicotiana tabacum*

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Abstract **In plants, the ABC transporter *PDR* (pleiotropic drug resistance) subfamily is composed of approximately 15 genes, few of which have been analyzed. We have identified *NtPDR3*, a *Nicotiana tabacum PDR* gene belonging to a cluster for which no functional data was previously available. NtPDR3 was found to be induced in suspension cells treated with methyl jasmonate, salicylic acid, 1-naphthalene acetic acid, or cembrene, a macrocyclic diterpene. In agreement with the identification of a putative iron deficiency element in the *NtPDR3* transcription promoter region, we found that iron deficiency in the culture medium induced NtPDR3 expression, thus suggesting a new function of the PDR transporter family.**
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Keywords: PDR; ABC transporter; Tobacco BY2; Methyl jasmonate; Iron deficiency

1. Introduction

ATP-binding cassette (ABC) transporters have been described in all organisms and are found in most biological membranes, where they couple ATP hydrolysis to the transport of a wide range of substrates across the membrane, generally against a concentration gradient [1].

Among the various ABC subfamilies, the PDR subfamily is only found in plants and fungi and has been mainly characterized in yeast (reviewed in [2,3]). Few plant PDR transporters have been characterized at the functional level [4,5]. The first was SpTUR2, from the aquatic plant *Spirodela polyrrhiza*, expression of which is induced by cold stress, NaCl, and treatment with drugs, including abscissic acid [6]. The *Nicotiana plumbaginifolia* ortholog, NpPDR1 (formerly NpABC1), is induced by sclareol, a diterpene involved in plant defense and there are indications that this metabolite is also transported by NpPDR1 [7]. This is also the case of SpTUR2 and the *Arabidopsis* ortholog, AtPDR12 [8,9]. Other indications of the involvement of PDR transporters in plant defense come from the induction of their expression by defense signaling molecules, such as methyl jasmonate (MJ) or salicylic acid, or directly upon pathogen inoculation [9–11]. Moreover, preventing *NpPDR1* expression by RNA silencing causes *N. plumbaginifolia* plants to become sensitive to the fungus, *Botrytis cinerea* [11]. *NtPDR1*, another closely related gene, was identified in tobacco bright yellow-2 (BY2) suspension cells after treatment with MJ and various elicitors [12]. The role of PDR genes might go beyond plant defense, since expression of *AtPDR12* is enhanced in plants by the lead and knockout *atpdr12* lines are more sensitive to lead [13]. Finally, in rice, *OsPDR9* expression is related to perturbation of the root environment, expression being triggered by plant growth hormones and general redox status changes in the roots [14].

All *PDR* genes for which functional data are currently available belong to the same cluster (Fig. 1). Since MJ is involved in many physiological processes, including plant development and stress responses [15] and activates genes involved in the biosynthesis of secondary metabolites, such as alkaloids, quinones, phenylpropanoid, and terpenes [16,17], we hypothesized that MJ might enhance the synthesis of other PDR transporters to facilitate metabolite excretion. Here, we report the identification, sequencing, and characterization of *NtPDR3*, which belongs to a previously non-characterized cluster, and show that its expression is strongly induced by iron deficiency.

2. Materials and methods

2.1. Plant material

N. tabacum BY2 [18] suspension cells were grown in MS medium (MP-Biomedicals, #2610024) supplemented as described in [10] with agitation (100 rpm) in the dark at 25 °C. Every week, 5 ml was reinoculated into 200 ml of fresh medium. Iron-lacking MS medium was prepared according to [19] without adding Fe^{2+}-EDTA.

2.2. Drug treatment of BY2 cells

Test chemicals were added to 3.5-day-old cultures. Cells were collected at intervals by filtration, washed with two volumes of 20 mM KCl, 5 mM Na-EDTA, 10 mM Tris (HCl), pH 8.0.

2.3. RNA isolation, Northern blotting and RT-PCR

RNA was isolated using a standard guanidine thiocyanate method. RT-PCR and Northern blotting were performed using standard methods. RT-PCR fragments were obtained with the following primers: 5′-CCCAAATGGTGGATCTGGTTC-3′ and 5′-CCATAACCTTATTTTCCCCC-3′ (*NtPDR3*: nucleotides 4041–4388); 5′-TCTTTGCTGGTGTTGGTGAA-3′ and 5′-TGAGCTCATCCATACCCAAA-3′ (*ATP2-1*: nucleotides 782–1432 [20]). These PCR fragments were cloned and labeled with ^{32}P-dCTP by random priming for Northern blotting.

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Abbreviations: ABC, ATP-binding cassette; MJ, methyl jasmonate; BY2, bright yellow-2

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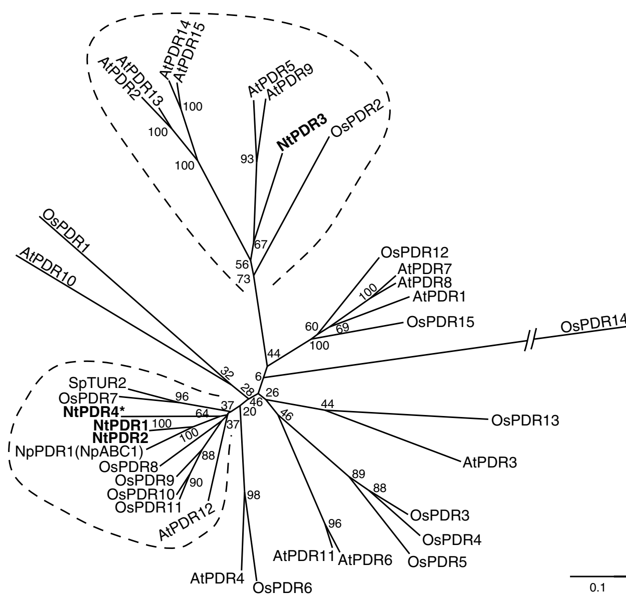

Fig. 1. Phylogenetic analysis of plant PDRs. Boot-straps and phylogenetic analysis were performed using Clustal W 1.83 [33] using amino acid sequences of PDRs from *Arabidopsis* [4] and *Oryza sativa* [34], SpTUR2 (CAA94437), NpPDR1 (formerly NpABC1, CAC40990), NtPDR1 (BAB92011) and NtPDR2 (BAD07484). At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Sp, *Spirodela polyrrhiza*; Np, *Nicotiana plumbaginifolia*; Nt, *Nicotiana tabacum* (in bold). (*) indicates that only the NtPDR4 C-terminal region (486 residues) was used in the alignment. OsPDR14 is represented at one-third of its real distance. Dashed lines indicate NtPDR1 and NtPDR3 clusters.

2.4. Cloning of PDR genes

cDNAs were generated from poly(A) RNA from cells treated for 4 h with 500 μM MJ. Two degenerate primers were used to amplify the 3′ part of the gene: (CysThrHisIleGlnProSer) MB1 (5′-AT[A/T/C]AT[A/T/C]TT[C/T]ATGGACGAACC-3′) and MB2 (5′-AT[A/T/C]AT[A/T/C]TT[C/T]ATGGACGAGCC-3′). A second nested-PCR was performed using oligo(dT) and four degenerate primers (AsnProAla ThrTrpMet): ED1 (5′-AA[C/T]CC[A/T/G/C]GC[A/T/G/C][A/G]CAT GGATG-3′), ED2 (5′-AA[C/T]CC[A/T/G/C]GC[A/T/G/C][A/G]CTT GGATG-3′), ED3 (5′-AA[C/T]CC[A/T/G/C]GC[A/T/G/C][A/G]CG TGGATG-3′), and ED4 (5′-AA[C/T]CC[A/T/G/C]GC[A/T/G/C][A/G]CCTGGATG-3′). The resulting PCR products were cloned into the pGEM T-easy vector (Promega). The *NtPDR3* 5′ end was obtained by dCTP tailing using a terminal transferase (Roche) followed by successive nested PCRs using oligo(dG) and specific *NtPDR3* primers.

A 760 bp fragment of the *NtPDR3* promoter was obtained by inverse PCR using two sets of *NtPDR3* internal nested primers and *Bcl*I-digested and religated DNA.

The accession numbers are AJ831379 (cDNA) and AM050393 (genomic) for *NtPDR3* and AJ831380 (partial cDNA) for *NtPDR4*.

2.5. Antibody preparation

Synthetic peptides corresponding to PDR sequences (see Section 3) were coupled to BSA (Imject Maleimide Conjugation® kit, Pierce) and used to immunize rabbits. Antibodies were purified from the antiserum using a SulfoLink® kit (Pierce).

2.6. Protein analysis and immunodetection

BY2 cell microsomal fractions and plasma membrane-enriched fractions were prepared as described previously [7]. SDS–PAGE and Western blotting were performed using standard methods. The antibodies used were anti-PDR or PDR3 (this paper) or anti-H^+-ATPase [21].

3. Results

3.1. MJ induction of PDR proteins

Comparison of the SDS gel pattern of microsomal and plasma membrane fractions of BY2 cells with and without 16 h treatment with 250 μM MJ revealed in the treated samples the presence of variant proteins (Fig. 2A), including one of 160 kDa, a size compatible with a full-size ABC transporter. Western blots using anti-PDR antibodies raised against a 22-amino acid sequence highly conserved in plant PDRs (GRTVVCTIHQPSIDIFEAFDEL, position 1039–1060 in NpPDR1) showed that, in the treated cells, a band of the same size was detectable in the microsomal fraction and strong in the plasma membrane fraction (Fig. 2B). An increase in the MJ concentration from 50 nM to 250 μM resulted in a progressive increase in the signal (Fig. 2C). Using 250 μM MJ, the increase was seen within 3 h and remained stable over 10 h (Fig. 2D). These results show that MJ rapidly induces the expression of one or several PDRs localized in the plasma membrane.

3.2. Sequence and phylogenetic analysis of MJ-induced tobacco PDR genes

Using degenerate primers corresponding to conserved *PDR* sequences, four *PDR* partial sequences were obtained after RT-PCR using RNA from MJ-treated samples, two of which corresponded to the previously described *NtPDR1* [12] and *NtPDR2* [22], two very closely related genes; the other two were named *NtPDR3* and *NtPDR4*.

Full-length *NtPDR3* cDNA was obtained by RACE and RT-PCR. The putative translation initiator codon (Fig. 3) was identified by its favorable ATG context (aaaATGGC) [23] and the presence of an in-frame upstream stop codon (TAA) at position −39. The cDNA contained a 4305 bp open reading frame coding for a protein of 163 330 Da with a typical PDR organization.

Phylogenetic analysis revealed that *NtPDR4* belongs to the same cluster as *NtPDR1* (Fig. 1). In contrast, *NtPDR3* (53% identity at the amino acid level with *NtPDR1*) clearly belongs to another, as yet uncharacterized, cluster containing several *Arabidopsis* genes and a single rice gene.

A 760 bp fragment encompassing the putative *NtPDR3* promoter region was obtained by inverse-PCR (Fig. 3). A putative TATA box was found at position −194 to −186 upstream of the ATG initiation codon. A search of a regulatory DNA sequence database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) identified an activation sequence-1 (*as-1*) box (−149 to −130), an iron deficiency element-1 (IDE-1) box (−182 to −167), and a general transcription activator (*Gmlbc3*) box (−99 to −74).

3.3. Expression of NtPDR3

MJ induction of *NtPDR3* and *NtPDR4* was monitored by Northern blotting using 3′ specific probes. *NtPDR3* transcripts were observed after 2 h treatment with 250 μM MJ (Fig. 4), increased up to 6 h, then rapidly decreased and were undetectable after 10 h, while the control gene *ATP2-1* was evenly expressed. No signal was detected using the *NtPDR4* probe (data not shown), indicating that this gene is expressed at a low level. In order to specifically detect expression of NtPDR3 protein, antibodies were raised against a 16-amino acid

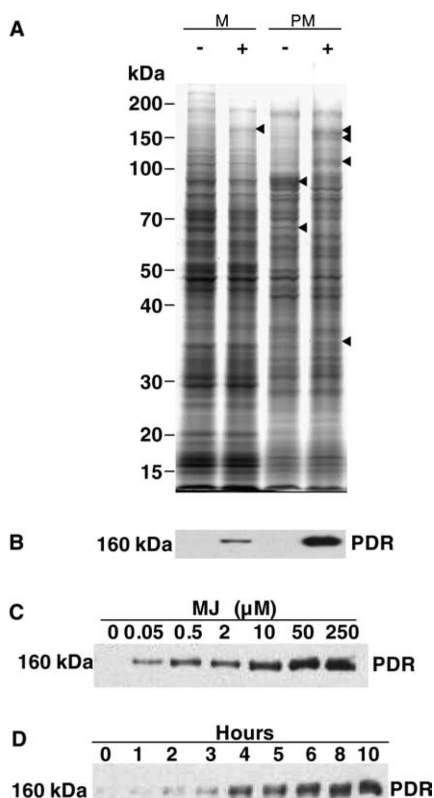

Fig. 2. Expression of BY2 cell membrane proteins after MJ treatment. (A) SDS–PAGE analysis (Coomassie blue staining) of microsomal (M) and plasma membrane (PM) fractions (20 μg of protein) prepared from untreated (−) BY2 cells or after 16 h treatment with 250 μM MJ (+). Arrow-heads indicate additional bands. (B) Samples from (A) analyzed by Western blotting using anti-PDR antibodies. (C) Western blotting analysis of PDR proteins in the BY2 cell microsomal fraction (20 μg) after 16 h treatment with the indicated MJ concentration. (D) Western blotting of PDR proteins in a BY2 cell microsomal fraction (20 μg) after treatment for the indicated time with 250 μM MJ.

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TGATCAGGCCTTACTTAGATTATGTAGTAGTTGGCTGTTCTTTCACTGAT -709
AAATGTTCATTTGATTGATACTAATATAGTTTTGCTGTGTTGTAGTACTG -659
TAATATTCATCTTCCTGGGGACCTTCTATTTAGCTGGGATGTCTCACGCC -609
TTTACAAGAGCTGACATACAGAACTCACCCTCCCCCACCCACCTCCCGCC -559
AACTACAAAGAATAGTTGGAATATTATAATACATGCCATAAACAAAGCAG -509
TAACGGGCATGGACCCAGCTATAATGGAAGCTCTATTGTAGATAAAGGAA -459
GACCCTAGCTCCTAGTTTAAGTCTTTGGAACTTTGGGAACTTATGGAACT -409
TACATGTCAGAAACCTTAGAATAATCATTTCTTTTTTGTCCTCCTAGGAA -359
AGTCAACTGACTCTGTCCCTTGAGGAGTTAGAGAAATGTGCAGCACTCTT -309
TTGTTTATTCAAAGATTTTTCTTTTGTTATTCTCCATAAACAAGCCTTAT -259
TTCATTTATTATCAACTGTAAACATCTGTTTACTCTCTAGGTATGTCTCT -209

                                ****** ****** **
TTAATTTACGTTATCTATATAAAGAAATCAAGAATGCTTTTATTCATAAA -159
                TATA-box        IDE1-box
         **** **** * **** ***
AAAAATGAATGATGTATTTCCTTACCTCATCTATGTTACGGTCTAAATTT -109
               as-1 box
                 *  ********** *** *
AGCTGTTCAAGCAAGCAAGAATATTTTATGTTAGACATGCACGGTGCACC -59
                            Gmlbc3 box
CCTCTTTGTTTTCTCCCTATAAATACAGCTTTGGAGCGGAAAATATTTTA -9
                   STOP
CACCAAAAATGGCTCAGTTGGTTGGTTCAGATGAAATAGAGTCATTTAGA +42
         M  A  Q  L  V  G  S  D  E  I  E  S  F  R
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Fig. 3. Sequence analysis of the putative *NtPDR3* promoter region. Nucleotides are numbered relative to the adenine of the presumed *NtPDR3* translation start codon. The coding sequence is in bold with the corresponding amino acid residues below. The *Bcl*I site used in inverse-PCR is underlined. Putative regulatory elements and a putative TATA box are boxed; (*) indicates nucleotides that match the consensus. Arrows indicate the *as-1* TGA motif in the forward and reverse orientation. A stop codon (TAA) in-frame with the first methionine is boxed with dashed lines. The arrow-head indicates the 5′ end of the cDNA.

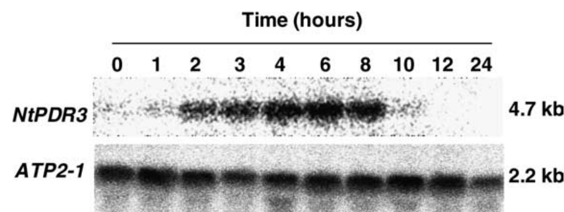

Fig. 4. MJ induction of *NtPDR3* transcription. RNA was prepared from BY2 cells treated for the indicated time with 250 μM MJ and analyzed (20 μg) by Northern blotting using the indicated probes.

sequence in a highly divergent PDR region (FRSNSALSASQKDDAV; residues 35–50), then BY2 cells were treated for 16 h with various compounds and NtPDR3 expression monitored by Western blotting (Fig. 5A). Ethanol (0.5%) slightly induced NtPDR3 expression. MJ, 1-naphathalene acetic acid and cembrene (dissolved in ethanol) induced NpPDR3 to a larger extent, unlike gibberellic acid and the diterpene sclareolide. Salicylic acid (dissolved in water) also induced NpPDR3 expression while abscissic acid, nicotine and NaCl had no effect. As a control, antibodies against the PDR conserved region gave a stronger signal with MJ and sclareolide, probably accounting for NpPDR1 expression [11]. Induction of NpPDR3 expression by cembrene, 1-naphthalene acetic acid and salicylic acid was confirmed by RT-PCR using *NpPDR3*-specific primers (Fig. 5B).

The presence of an IDE-1 element in the *NtPDR3* promoter prompted us to investigate its induction pattern on iron deprivation. Chelation of divalent ions by addition of EDTA to the growth medium triggered NtPDR3 expression in BY2 cells (Fig. 5C). Since EDTA chelates ions other than iron, iron deficiency-specific NtPDR3 induction was demonstrated by transfer of BY2 cells to iron-free medium while no expression was observed when iron was included in the medium (Fig. 5D). RT-PCR using *NtPDR3* specific primers confirmed these data (Fig. 5E).

4. Discussion

All previously characterized *PDR* genes belong to the same cluster and all seem to be involved in plant defense. Here, we characterized *NtPDR3*, a gene belonging to a cluster previously uncharacterized at the functional level. *NtPDR3* upregulation by MJ and salicylic acid indicates that this gene might also be involved in plant defense. Sclareolide and cembrene belong to two structurally different diterpene classes, the polycyclic labdanes and the macrocyclic duvanes, respectively, several of which possess anti-fungal properties in *Nicotiana* species [24]. While NpPDR1 expression is induced by sclareolide [7], but only little by cembrene (data not shown), NtPDR3 expression was induced by cembrene, but little by sclareolide (Fig. 5). Thus, these two PDR genes, which belong to two different clusters, are controlled by different diterpene classes. Besides inducing NpPDR1 expression, sclareol is a substrate of this

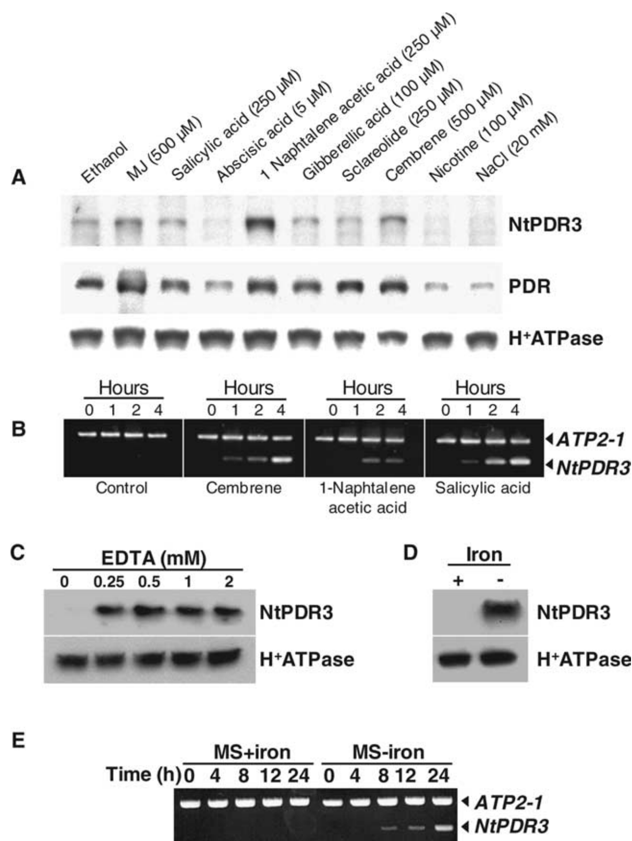

Fig. 5. Induction of NtPDR3 expression by various chemicals or iron deficiency. (A) Microsomal fractions were prepared from BY2 cells 16 h after addition of the indicated compounds and analyzed by Western blotting (20 μg) using NtPDR3, PDR or H^+-ATPase antibodies. MJ, 1-naphthalene acetic acid, gibberellic acid, sclareolide and cembrene were added as ethanol solutions (0.5% ethanol final concentration). (B) RT-PCR of *NtPDR3* transcripts from BY2 cells grown for the indicated periods of time in the presence of 500 μM cembrene, 250 μM 1-naphthalene acetic acid or 250 μM salicylic acid. *ATP2-1* was used as a control. (C) Western blotting of a microsomal fraction (40 μg) from BY2 cells 48 h after addition of the indicated ETDA concentration. (D) Western blotting of plasma membrane fractions (20 μg) from BY2 cells grown for 48 h in a synthetic MS medium lacking iron. (E) RT-PCR of *NtPDR3* transcripts from BY2 cells grown for the indicated periods of time in an iron-free medium (−iron) or in the same medium supplemented with 100 μM Fe^{2+}-EDTA (+iron).

transporter [7], so it will be interesting to determine whether cembrene, in addition to triggering NtPDR3 expression, is also a substrate for the encoded transporter.

NtPDR3 expression was induced by iron deficiency in the culture medium. In agreement with this observation, we identified a putative IDE-1 element in the *NtPDR3* promoter region. IDE-1 elements are found in many iron-deficiency inducible promoters [25,26]. We could therefore propose that *NtPDR3* is involved in iron homeostasis. An alternative hypothesis would be that iron deficiency upregulates iron transporters, some of which are known to be leaky for Cu^{2+} and Zn^{2+} [27,28]. Intracellular accumulation of these ions would result in cell intoxication and indirectly induce expression of *NtPDR3*. We can rule out this hypothesis since EDTA also chelates these divalent cations and still induced *NtPDR3* expression.

The observation that NtPDR3 was enriched in the plasma membrane fraction supports the hypothesis that it is involved in transport in or out of the cell, rather than in intracellular compartmentalization like IDI7, a vacuolar ABC transporter belonging to the TAP subfamily [29]. Although iron is abundant in soil, it is mainly present in its less soluble oxidized form (Fe^{3+}) that is poorly available to plants. Iron uptake by non-grass plant roots is improved by acidification of the rhizosphere: Fe^{3+} is more soluble at low pH and is chelated by organic acids that are secreted, Fe^{3+}-chelates are then reduced to Fe^{2+} by ferric-chelate reductase, and the ferrous iron is transported into the cell through Fe^{2+}-transporters (reviewed by [30]). Besides root uptake, other steps are involved in long-distance iron transport in the plant, such as xylem loading in the root, phloem loading in the source tissues, and uptake by all plant cells. NtPDR3 might therefore be involved in any of these steps, either directly in iron transport, or indirectly, such as in the transport of organic acids in the rhizosphere or of nicotianamine, which has been proposed to chelate iron in the phloem [31].

NtPDR3 activation by 1-naphthalene acetic acid might be related to iron deficiency. Indeed, an involvement of auxin in root epidermis cell development of iron-deficient plants was inferred from phenotypical analysis of hormone-related *Arabidopsis* mutants and from the application of auxin antagonists [32].

The relationship, if any, between cembrene and iron deficiency is not clear. However, several ABC transporters transport many different substrates with unrelated structures and might thus be involved in different physiological roles. Detailed examination of NtPDR3 expression in plant tissues and genetic approaches aimed at modifying its expression are needed to understand the precise roles of NtPDR3 in the plant.

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