A Gain-of-Function Mutation in the Arabidopsis Pleiotropic Drug Resistance Transporter PDR9 Confers Resistance to Auxinic Herbicides

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Arabidopsis (Arabidopsis thaliana) contains 15 genes encoding members of the pleiotropic drug resistance (PDR) family of ATP-binding cassette transporters. These proteins have been speculated to be involved in the detoxification of xenobiotics, however, little experimental support of this hypothesis has been obtained to date. Here we report our characterization of the Arabidopsis PDR9 gene. We isolated a semidominant, gain-of-function mutant, designated pdr9-1, that exhibits increased tolerance to the auxinic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Reciprocally, loss-of-function mutations in PDR9 confer 2,4-D hypersensitivity. This altered auxin sensitivity defect of pdr9 mutants is specific for 2,4-D and closely related compounds as these mutants respond normally to the endogenous auxins indole-3-acetic acid and indole-butyric acid. We demonstrate that 2,4-D, but not indole-3-acetic acid transport is affected by mutations in pdr9, suggesting that the PDR9 transporter specifically effluxes 2,4-D out of plant cells without affecting endogenous auxin transport. The semidominant pdr9-1 mutation affects an extremely highly conserved domain present in all known plant PDR transporters. The single amino acid change results in increased PDR9 abundance and provides a novel approach for elucidating the function of plant PDR proteins.

Auxin regulates numerous aspects of plant growth and development including embryogenesis, lateral root formation, vascularization, and tropic growth responses (Woodward and Bartel, 2005). While endogenous auxin plays a critical role in normal plant growth and development, the application of high doses results in phytoxicity. This observation led to the development of several synthetic auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D) as the first successful selective herbicide. 2,4-D and other auxinic herbicides are largely specific to dicots, however, the mechanisms underlying this selectivity are poorly understood. Some 60 years after its discovery, 2,4-D remains the most widely used herbicide worldwide (www.epa.gov). While recent decades have seen the development of several herbicide-resistant crop varieties that have profoundly altered agricultural practices, such is not the case for 2,4-D and other auxinic herbicides. This is complicated in part by the essential role of endogenous auxin (indole-3-acetic acid [IAA]) in plant growth and development. Mutant or transgenic varieties resistant to 2,4-D also display altered response to IAA and consequently exhibit abnormal development. Insight into the mechanism underlying this phenomenon was recently provided by the demonstration that IAA and 2,4-D bind a common receptor to regulate auxin signaling (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005).

Common strategies to achieve herbicide tolerance through genetic and transgenic approaches are typically aimed at identifying mutant target proteins unaffected by the herbicide or the metabolic detoxification/degradation of the compound. An additional approach was recently suggested by Windsor et al. (2003) involving herbicide detoxification by efflux facilitated by plant ATP-binding cassette (ABC) transporters. These proteins, which are found in all living organisms, mediate the translocation of a wide range of structurally unrelated molecules across biological membranes (Higgins, 1992). All functional ABC transporters have a basic structural organization consisting of two hydrophobic transmembrane spanning domains (TMDs) and two hydrophilic nucleotide-binding domains (NBDs). The NBDs contain a highly conserved 200-amino acid region consisting of the Walker A and Walker B boxes (Walker et al., 1982), separated by approximately 120 amino acids containing the ABC signature motif (Bairoch, 1992). Members of ABC transporter family are categorized by the configuration of TMDs and NBDs, and are referred to as half-size or full-size transporters based on the number (one or two) of TMD/NBD modules they contain (Higgins, 1992).

In Arabidopsis (Arabidopsis thaliana), approximately 130 genes encoding ABC transporters have been identified in the completed Arabidopsis genome (Sanchez-Fernandez et al., 2001; Martinou et al., 2002; Schulz and Kolukisaoglu, 2006). Fifty four of these genes belong to the full-size category and are classified into three
groups: P glycoproteins (PGPs; also referred to as multidrug resistance [MDR] transporters), MDR-associated proteins (MRPs), and pleiotropic drug resistance (PDR) transporters (Theodoulou, 2000; Sanchez-Fernandez et al., 2001; van den Brule and Smart, 2002; Crouzet et al., 2006). Arabidopsis PDR1, PDR2, and PDR3 transport glutathione conjugates of endogenous and artificial substrates into vacuoles (Lu et al., 1997, 1998; Tommasini et al., 1998), and PDR4 and PDR5 have been implicated in the complex regulation of stomatal aperture and guard cell ion flux (Gaedeke et al., 2001; Klein et al., 2003, 2004). The majority of plant PGP/MDRs characterized to date have been implicated in the transport of auxin (Multani et al., 2003; Geisler and Murphy, 2006). Recently, Arabidopsis PGF1 and PGP19 have been reported to mediate the ATP-dependent cellular efflux of natural and synthetic auxins as well as oxidative auxin breakdown products in Arabidopsis protoplasts and whole plants (Geisler et al., 2003, 2005). Additionally, Arabidopsis PGP4 has been suggested to function primarily in the uptake of redirected or newly synthesized auxin in epidermal root tip cells (Santelia et al., 2005; Terasaka et al., 2005).

In contrast to the PGP class of ABC transporters, the plant PDR subfamily has received considerably less attention. Genetic studies on Arabidopsis PDR1 and its likely homologs in Nicotiana plumbaginifolia and Spirodela polyrrhiza have implicated this transporter in the efflux of salicaleor, an antifungal diterpene (Jasinski et al., 2001; van den Brule et al., 2002; Campbell et al., 2003; Stukkens et al., 2005). An additional connection between PDR-type transporters and defense response is provided by recent studies of atpdr8 mutants, which exhibit increased cell death associated with the hypersensitive response following pathogen infection (Kobae et al., 2006; Stein et al., 2006). AtPDR12 has also recently been implicated in lead detoxification (Lee et al., 2005).

Here we report our characterization of the Arabidopsis PDR9 transporter. We identified the semidominant pdr9-1 mutation as conferring increased 2,4-D tolerance in a genetic screen to isolate mutations enhancing the relatively weak auxin response defect conferred by the tir1-1 mutation (Gray et al., 2003; Chuang et al., 2004; Quint et al., 2005). Reciprocally, recessive loss-of-function alleles of PDR9 result in 2,4-D hypersensitivity, indicating that pdr9-1 is a gain-of-function mutation conferring increased activity to the protein. Further analysis demonstrated that PDR9 is involved in the translocation of not only 2,4-D but also other members of the phenoxylalkanoic acid family of herbicides as well as the polar auxin transport inhibitor, naphthylphthalamic acid (NPA). In contrast, we find that PDR9 is not involved in the transport of the native auxin, IAA.

RESULTS

Identification of the eta4 Mutation

We have previously described a genetic screen designed to identify mutations that enhance the relatively weak auxin resistance phenotype of tir1-1 seedlings. Several enhancer of tir1-1 auxin resistance (eta) mutants were isolated in this screen, including ETA3/SGT1b (Gray et al., 2003), ETA2/CAND1 (Chuang et al., 2004), and ETA1/AXR6/CUL1 (Quint et al., 2005), all three of which encode components of SCF[TIR1] pathway. The eta4 tir1-1 M2 plant was backcrossed to Columbia (Col) and eta4 single mutants isolated by PCR-based genotyping of TIR1. When eta4/eta4 TIR1+/-TIR1+ plants were backcrossed again to Col, 233/317 F2 seedlings displayed resistant root growth on 0.075 μM 2,4-D, indicating that the eta4 mutation confers a dominant auxin-resistant phenotype (1:3; χ² = 0.85). The amount of 2,4-D-mediated inhibition of root growth varied considerably among the resistant progeny, and 2,4-D dose-response studies revealed that eta4 actually behaves in a semidominant fashion (Fig. 1, A and B).

Despite the finding that eta4/eta4 seedlings exhibit stronger 2,4-D resistance than tir1-1 mutants (Fig. 1B), unlike tir1-1, the eta4 mutation did not confer a reduction in lateral root development (Fig. 1C). Furthermore, eta4 mutants did not exhibit any of the auxin-related developmental defects, such as reductions in stature and apical dominance characteristic of many auxin-response mutants (Woodward and Bartel, 2005). Instead, eta4 plants appeared indistinguishable from wild-type controls at all stages of development (data not shown).

The finding that the eta4 mutation conferred 2,4-D-resistant root growth but no detectable auxin-related phenotypes, suggested that the eta4 defect may be specific to the synthetic auxin, 2,4-D. While no auxin-response mutants have been described that discriminate between native and synthetic biologically active auxins, there are well-characterized differences in the transport of IAA, 2,4-D, and naphthylacetic acid (NAA; Morris et al., 2004). For example, while 2,4-D and IAA are taken up by the putative influx carrier AUX1 (Marchant et al., 1999), only IAA is an efficient substrate for the efflux carriers of the polar auxin transport system (Morris et al., 2004). We compared the effects of exogenous 2,4-D, IAA, and NAA on root growth of eta4 seedlings and found that eta4 auxin resistance was entirely 2,4-D specific (Fig. 1D). Likewise, whereas IAA and NAA promoted lateral root development equally well in eta4 and wild-type seedlings, eta4 mutants exhibited a striking reduction in 2,4-D-induced lateral root formation (data not shown). The 2,4-D-specific auxin resistance of eta4 was also examined at the level of gene expression using the auxin-responsive reporter construct, BA-β-glucuroni-dase (GUS; Oono et al., 1998). Homozygous Col[BA-GUS] and eta4[BA-GUS] seedlings were treated with 1 μM auxins for 6 h. As reported in Oono et al. (1998), strong induction of GUS activity was observed in the proximal region of the root elongation zone when Col seedlings were treated with auxins (Fig. 1E). While eta4 seedlings exhibited a wild-type-like response to IAA and NAA, only faint GUS staining was detected
when eta4 seedlings were treated with 2,4-D (Fig. 1E), further demonstrating the 2,4-D-specific nature of the eta4 mutant.

**ETA4 Encodes the PDR9 Protein**

A map-based cloning strategy was used to isolate the ETA4 gene. The eta4 mutation was initially mapped between nga162 and nga6 on the south end of chromosome 3. Additional mapping narrowed the location of eta4 to an approximately 200 kb interval. Inspection of candidate loci within this interval revealed the presence of two ABC-type transporter genes, one exhibiting similarity to yeast (*Saccharomyces cerevisiae*) *PDR5* (At3g53480), and a second related to the human breast cancer resistance protein, BCRP (At3g53510). Our finding that the eta4 defect is 2,4-D specific, together with recent findings implicating ABC transporters in auxin transport (Noh et al., 2001; Geisler et al., 2005; Santelia et al., 2005), and studies in yeast demonstrating that loss of *pdr5* results in 2,4-D hyper-sensitive growth arrest (Teixeira and Sa-Correia, 2002), led us to sequence the coding regions of these two genes from eta4 plants. We detected no sequence difference between eta4 and wild type for At3g53510, but we did identify a 1-bp substitution (G to A) in the

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**Figure 1.** Characterization of the eta4 mutant. A, Seedlings were grown on unsupplemented nutrient medium for 4 d and then transferred to medium containing 0.075 μM 2,4-D and grown for an additional 4 d. Asterisks indicate the position of the root tip at the time of transfer. Genotypes were confirmed by sequencing of PCR products. Size bar = 5 mm. B, Inhibition of root elongation by increasing concentrations of 2,4-D. Data points describe the mean of 10 seedlings. Standard deviations for all data points were ≤10% of the mean. C, Lateral root (LR) initiation was assessed in 10-d-old seedlings grown on unsupplemented nutrient medium (n = 10). D, Inhibition of root elongation by increasing concentrations of IAA and NAA. Data points represent the mean from 10 seedlings. E, Auxin-dependent GUS expression in Col and eta4 seedlings carrying the BA-GUS reporter construct. Each homozygous BA-GUS line was incubated in liquid ATS medium with or without 1 μM auxins for 6 h and then stained for 16 h.
18th exon of At3g53480 (Fig. 2A). At3g53480 has been designated as PDR9 among 15 PDR genes identified in Arabidopsis genome (van den Brule and Smart, 2002). Thus, we designated eta4 as pdr9-1. PDR9 contains 23 exons and encodes a 1,450-amino acid protein.

A search of the SALK Institute’s SIGnAL T-DNA database (Alonso et al., 2003) identified multiple lines containing a T-DNA insertion within the PDR9 locus (Fig. 2A). We confirmed the T-DNA insertion site in the third exon of SALK_050885, which we designated as pdr9-2. RNA gel-blot analysis detected a 4.5 kb transcript present in RNA prepared from wild-type seedlings and absent from homozygous pdr9-2 samples, suggesting that pdr9-2 is a likely null allele (Fig. 2B).

We next examined the affect of 2,4-D on root growth of pdr9-2 seedlings. As shown in Figure 2C, pdr9-2 plants showed clear hypersensitivity to 2,4-D, with root growth almost completely inhibited at 0.01 μM. Consistent with this finding, pdr9-2 seedlings also exhibited increased sensitivity to the 2,4-D-induced expression of the DR5-GUS (Ulmason et al., 1997) reporter of auxin-regulated gene expression (Fig. 2D). IAA-mediated DR5-GUS expression, however, was unaffected in the pdr9-2 mutant (Fig. 2D). The contrasting 2,4-D phenotypes conferred by the semidominant pdr9-1 mutation and recessive pdr9-2 null mutation demonstrate that pdr9-1 is a gain-of-function mutation. Like the pdr9-1 mutant, however, pdr9-2 plants exhibited no detectable growth or developmental phenotype.

PDR proteins are characterized by the possession of two predicted cytosolically oriented NBD domains linked to two multiple-pass hydrophobic TMDs in the arrangement NH2-NBD1-TMD1-NBD2-TMD2-COOH. A search of the ARAMEMON database predicted that PDR9 contains two sets of six-pass TMDs (Fig. 3A). The mutation identified in pdr9-1 causes an Ala to Thr substitution at position 1,034 within the highly conserved plant PDR signature sequence adjacent to the Walker B motif of NBD2 (Fig. 3B). The Walker A and Walker B motifs, which are needed for ATP binding and hydrolysis, are highly conserved, even in distinct types of ABC transporter family members. The entire adjacent 12-amino acid PDR signature motif encompassing the Ala-1,034 residue affected in pdr9-1 (Fig. 3B) is absolutely conserved in all 15 Arabidopsis PDR proteins as well as in nearly all other plant PDR proteins present in available databases including 20 of the 23 rice PDRs. In contrast, this domain is not highly conserved between plant PDRs and other types of plant ABC transporters, or even between plant and fungal PDR proteins (Fig. 3B). This extreme sequence conservation strongly suggests that this motif plays an important role in plant PDR function.

Expression Analysis of PDR9

We next analyzed PDR9 expression patterns by RNA gel-blot analysis of RNA prepared from various organs. The PDR9 mRNA was exclusively detected in roots (Fig. 4A). It has been reported that the expression of some ABC transporters, including the previously characterized Arabidopsis PDR12, is strongly induced by the application of their putative substrates (van den Brule and Smart, 2002; Lee et al., 2005). We therefore tested the possibility that PDR9 expression is induced by auxins (Fig. 4B). However, PDR9 expression was not affected by 2,4-D, IAA, or NAA treatments. The effect of auxin treatment was confirmed by the clear induction of IAA5 (Fig. 4B). We also examined the tissue-specific expression of PDR9 using transgenic plants carrying the PDR9 promoter fused with the
GUS reporter. Strong GUS activity was observed throughout the root, with expression highest in the cells of the lateral root cap and epidermal cells at the root tip (Fig. 4, C and D). The only shoot expression that was detectable was in the stipules (Fig. 4C, inset). Consistent with our reverse transcription (RT)-PCR studies, exogenous auxins did not result in increased PPDR9-GUS expression (data not shown).

Characterization of the PDR9 Protein

To characterize the PDR9 protein, we raised polyclonal antisera against a recombinant 6×His fusion protein containing the N-terminal 98 amino acids of PDR9. The α-PDR9 antisera detected a single band of approximately 160 kD in wild-type and pdr9-1 extracts prepared from Arabidopsis roots (Fig. 5A). This band was completely absent in pdr9-2 microsomal extracts, confirming that the antisera recognizes the PDR9 protein and that pdr9-2 is a likely null allele. Consistent with a predicted membrane localization, PDR9 was only detected in microsomal fractions. Interestingly, we observed a slight but consistent increase in PDR9 abundance in microsomal extracts prepared from pdr9-1 roots than from wild type (Fig. 5, A and B). Analysis of western blots of four independent sets of microsomal membrane preparations indicated that pdr9-1 roots contain approximately 1.8 ± 0.2-fold more PDR9 protein than wild-type controls. In contrast, we could detect no difference in PDR9 mRNA levels between wild type and pdr9-1 (Fig. 5B), suggesting that the mutation might confer increased protein stability, which could potentially account for its semidominant nature.

To further investigate the subcellular localization of PDR9, microsomal extracts were subjected to aqueous two-phase partitioning. PDR9 fractionated almost exclusively to the plasma membrane (PM) enriched upper phase (Fig. 5C), as did the known PM protein PGP4 (Terasaka et al., 2005). In contrast, the endoplasmic reticulum-associated protein, SEC12, was highly enriched in the lower phase. We obtained identical results in two-phase fractionations with pdr9-1 microsomes, suggesting that the mutant protein is not altered in its membrane localization (data not shown).
Effects of Additional Auxinic Compounds on pdr9 Mutants

The finding that ETA4 encodes a PDR-type ABC transporter that localizes to the PM and affects sensitivity to 2,4-D but not the endogenous auxin IAA suggested that PDR9 might be involved in the cellular detoxification of xenobiotics. This led us to test the effects of several additional herbicides on our pdr9 mutants to further examine specificity. Because of its widespread usage in the agricultural and horticultural fields, several 2,4-D-related compounds have been developed that exhibit auxin-like activities. For example, 2,4-D, 4-chlorophenoxy-acetic acid, 4-chloro-2-methylphenoxy acetic acid, and 2,4,5-trichlorophenoxyacetic acid all share a 4-chloro-phenoxy ring, but differ in terms of the number of chloride or methyl groups present. As observed with 2,4-D, the gain-of-function and loss-of-function pdr9 alleles conferred opposing phenotypes in root growth inhibition assays with these 2,4-D-related herbicides (Fig. 6, A–C). In contrast, wild-type and the pdr9 mutants exhibited similar sensitivity toward p-chlorophenoxyisobutyric acid, which has a 4-chloro-phenoxy ring with an isobutyric rather than an acetic acid group at position 1. This finding suggests that the acidic side chain may be an important determinant of specificity, however, it should be noted that the concentration of p-chlorophenoxyisobutyric acid necessary to inhibit root growth is considerably higher than these other auxins (Fig. 6D).

We also examined sensitivities to the IAA derivatives indole-butyric acid, 4-Cl-IAA, and 4,5-Cl-IAA. The responsiveness to these IAA-related compounds in pdr9-2 plants was essentially the same as wild type, suggesting that PDR9 is not involved in their transport. However, while pdr9-1 plants responded normally to IAA and indole-butyric acid, they were slightly resistant to 4-Cl-IAA and even more so to 4,5-Cl-IAA (Fig. 6, E–H). These results suggest that the gain-of-function pdr9-1 mutation might affect substrate recognition and confer a broader range of substrate specificity. We also tested several concentrations of NAA, dicamba, picloram, abscisic acid, cycloheximide, Cu²⁺, Co²⁺, and Zn²⁺, but detected no significant differences between the pdr9 mutants and wild type (data not shown).

pdr9-2 Is Hypersensitive to NPA

Although neither pdr9 allele conferred any change in sensitivity to IAA or NAA, we wanted to further examine the possibility that PDR9 might be involved in auxin transport since several recent studies have implicated PGP/MDR subfamily members of ABC transporters in polar auxin flow (Geisler et al., 2003, 2005; Terasaka et al., 2005). We analyzed the gravitropic response of both pdr9 alleles by plate rotation and root curling assays, but could detect no significant changes from wild type in the gravitropism response (data not shown). However, when pdr9-2 seedlings were grown in the presence of the polar auxin transport inhibitor NPA, we observed a dramatic increase in sensitivity compared to wild type. pdr9-2 seedlings exhibited agravitropic root and hypocotyl growth on concentrations of NPA as low as 0.05 μM, whereas wild-type and pdr9-1 seedlings were largely unaffected at this concentration (Fig. 7, A–F). We also examined the effect of NPA on root growth in quantitative root inhibition assays. pdr9-2 seedlings exhibited an approximately 100-fold increase in sensitivity compared to wild type and pdr9-1 (Fig. 7G).

Altered 2,4-D and NPA Accumulation in pdr9 Mutants

To confirm that the physiological responses observed in the pdr9 mutants were due to the altered transport of 2,4-D or NPA, we conducted accumulation assays using [¹⁴C]-2,4-D and [³H]-NPA. Based on the expression analysis of PDR9 (Fig. 4), root tips (apical 5 mm) were collected from 5-d-old seedlings and incubated in buffer containing labeled 2,4-D or NPA for 60 min. Following a brief rinse, the root tips were collected and radioactivity levels measured by liquid scintillation counting. Consistent with our physiological assays indicating that the pdr9 mutations confer altered 2,4-D sensitivity but do not affect IAA sensitivity, we observed significant hyperaccumulation and hypoaccumulation of [¹⁴C]-2,4-D in pdr9-2 and pdr9-1 roots, respectively (Fig. 8A), but no difference in [³H]-IAA accumulation (Fig. 8B). When incubated with [³H]-NPA, pdr9-2 roots accumulated dramatically more label than wild-type roots (Fig. 8C).
pdr9-1 root tips exhibited a slight, albeit statistically insignificant, reduction in [3H]-NPA. However, this minor difference between wild type and pdr9-1 was also seen with shorter labeling periods (15 or 30 min; data not shown), suggesting that NPA transport may also be affected by the pdr9-1 mutation.

**DISCUSSION**

**Mutations in PDR9 Alter 2,4-D, But Not IAA Sensitivity**

In the past 20 years, numerous mutants exhibiting altered auxin sensitivity have been described. Since both IAA and the synthetic auxin 2,4-D are recognized...
by the same receptors, the TIR1/AFB family of F-box proteins (Dharmasiri et al., 2005a, 2005b), all auxin signaling mutants described to date exhibit altered response to both of these auxins. In contrast, several studies have demonstrated that major differences exist in the transport of IAA and 2,4-D, with the latter being a poor substrate for the polar auxin transport system (Delbarre et al., 1996; Morris et al., 2004). Our complementary findings with gain- and loss-of-function pdr9 mutants accumulate less or more 2,4-D, respectively, than wild-type controls. Given these findings, together with the likely PM localization of PDR9 suggested by our microsome fractionation studies, it is likely that PDR9 acts as a 2,4-D pump capable of effluxing 2,4-D out of plant cells. While our 2,4-D accumulation assays do not directly discriminate between 2,4-D influx and efflux, since the pdr9-2 null mutant hyperaccumulates 2,4-D resulting in increased sensitivity to the herbicide, one would need to invoke a mechanism whereby PDR9 normally negatively regulates a protein that imports 2,4-D in order for influx to be altered in the pdr9 mutants. 2,4-D can enter plant root cells via the putative auxin influx carrier AUX1 (Marchant et al., 1999). However, PDR9-mediated negative regulation of AUX1 activity seems highly unlikely given that [3H]-IAA accumulation was identical in wild type and the pdr9 mutants.

We attempted to demonstrate that PDR9 can directly transport 2,4-D by expression in heterologous systems, but these experiments were unsuccessful, largely due to technical complications. First, we expressed PDR9 in yeast to try and complement the 2,4-D hypersensitivity conferred by a pdr5 mutation. However, PDR9 expression was somewhat toxic to yeast, conferring a severe slow-growth phenotype that made complementation difficult to ascertain. Additionally, membrane fractionation studies and expression of a PDR9-GFP

Using several physiological and molecular assays, we demonstrate that the pdr9-1 gain-of-function mutation specifically confers increased 2,4-D resistance. Reciprocally, the pdr9-2 null mutation confers 2,4-D hypersensitivity. These findings correlate precisely with [14C]-2,4-D accumulation assays that demonstrate the gain- and loss-of-function pdr9 mutants accumulate less or more 2,4-D, respectively, than wild-type controls. Given these findings, together with the likely PM localization of PDR9 suggested by our microsome fractionation studies, it is likely that PDR9 acts as a 2,4-D pump capable of effluxing 2,4-D out of plant cells. While our 2,4-D accumulation assays do not directly discriminate between 2,4-D influx and efflux, since the pdr9-2 null mutant hyperaccumulates 2,4-D resulting in increased sensitivity to the herbicide, one would need to invoke a mechanism whereby PDR9 normally negatively regulates a protein that imports 2,4-D in order for influx to be altered in the pdr9 mutants. 2,4-D can enter plant root cells via the putative auxin influx carrier AUX1 (Marchant et al., 1999). However, PDR9-mediated negative regulation of AUX1 activity seems highly unlikely given that [3H]-IAA accumulation was identical in wild type and the pdr9 mutants.

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Figure 7. pdr9-2 plants are hypersensitive to NPA treatment. A to F, Effects of NPA on seedling growth. Seedlings were grown in dark for 5 d with (B, D, and F) or without (A, C, and E) 0.05 μM NPA. A and B, Wild type. C and D, pdr9-1. E and F, pdr9-2. Size bar = 1 cm. G, Inhibition of root elongation by NPA. Seedlings were grown on unsupplemented nutrient medium for 4 d and then transferred to medium containing different concentration of NPA and grown for an additional 4 d. Data points are averages from 10 seedlings, sds for all data points were <10% of the mean.

Figure 8. Altered transport of 2,4-D and NPA in pdr9 mutants. Root tips from 5-d-old Arabidopsis seedlings were excised and incubated in buffer containing radiolabeled [14C]-2,4-D (A), [3H]-IAA (B), or [3H]-NPA (C), rinsed briefly, and analyzed by scintillation counting. Values are the means from four or five independent experiments, each performed in duplicate.
construct both indicated that PDR9 did not localize to the PM properly in yeast. Other investigators have encountered similar difficulties in expressing plant PDR proteins in yeast (van den Brule et al., 2002; Crouzet et al., 2006). We also expressed PDR9 in Xenopus oocytes. Although we did observe a low level of PDR9 expression, we were unable to convincingly demonstrate any difference in 2,4-D efflux between injected and un.injected oocytes.

Several recent findings have implicated members of the PGP/MDR subfamily of ABC transporters in polar auxin transport. However, none of our findings suggest that PDR9 plays a role in this important process. The pdr9 mutants exhibit normal IAA sensitivity, and are unaffected in auxin-mediated processes such as lateral root development and tropic growth responses. Furthermore, we show that the pdr9 mutations specifically affect the transport of 2,4-D and closely related synthetic auxins without altering IAA transport. Although the pdr9-2 mutant exhibits heightened sensitivity to the polar auxin transport inhibitor NPA, this is almost certainly due to the fact that this mutant hyperaccumulates NPA as we demonstrate in uptake assays with [3H]-NPA. This latter finding also demonstrates that plants can transport NPA and should be considered by those employing this inhibitor in auxin transport studies.

What Is the Cellular Role of PDR9?

A significant unresolved question from our study regards the normal cellular function of PDR9. Neither the pdr9-1 nor pdr9-2 mutants exhibit any growth or developmental phenotype. We addressed the possibility that this might be attributable to genetic redundancy by examining T-DNA insertion mutants of the most closely related Arabidopsis PDR family member, PDR5. Like the pdr9 mutants, neither pdr5 mutant (SALK_002380 and SALK_035106) exhibited a discernible growth phenotype (H. Ito and W.M. Gray, unpublished data). However, unlike pdr9-2, the pdr5 mutants did not exhibit altered sensitivity to 2,4-D or NPA. Furthermore, pdr5 pdr9-2 double mutants behaved exactly like pdr9-2 single mutants in 2,4-D dose-response assays, suggesting the two proteins are functionally distinct (H. Ito and W.M. Gray, unpublished data).

Several recent findings have implicated AtPDR8, AtPDR12, and NpPDR1 in plant defense responses (Campbell et al., 2003; Stukkens et al., 2005; Kobae et al., 2006; Stein et al., 2006). These studies suggest that these transporters may extrude secondary metabolites with antimicrobial activity as part of the plant’s response to pathogen infection. Most notably, atpdr8/pen3 mutants were isolated in a genetic screen for mutations conferring reduced resistance to the barley powdery mildew Blumeria graminis, while NpPDR1-silenced tobacco (Nicotiana tabacum) plants exhibit reduced resistance to Botrytis cinerea (Stukkens et al., 2005). Such studies suggest that PDR9 might also be involved in defense responses. We note that numerous cis-acting elements implicated in defense responses, including nine putative WRKY transcription factor-binding sites, are present within 650 bases of sequence upstream of the PDR9 transcription start site (W box; http://www.dna.afrc.go.jp/PLACE/). Strong expression in the lateral root cap and epidermis also suggests that PDR9 might function in communication between plant roots and other organisms in the rhizosphere. Additionally, PDR9 has been identified as being strongly inducible by the defense elicitor salicylic acid (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004), however we were unable to verify this result in RT-PCR assays or with our PpPDR9-GUS reporter lines (H. Ito and W.M. Gray, unpublished data).

If the ability to act as a transporter of 2,4-D and related phenoxy acids is indicative of the endogenous substrate of PDR9, several phenolic secondary metabolites with antimicrobial activity including trans-cinnamic, ferulic, o-coumaric, and vanillic acid have all been identified in Arabidopsis root exudates following treatment with various pathogen elicitors (Walker et al., 2003). All of these compounds inhibit root growth at micromolar concentrations, however, we could detect no significant difference in response between wild type and the pdr9 mutants (H. Ito and W.M. Gray, unpublished data). Unfortunately, the root-specific expression pattern of PDR9 combined with the absence of an established quantitative assay for susceptibility to root-invading pathogens preclude us from readily examining the pdr9 mutants for altered pathogen resistance.

The Gain-of-Function pdr9-1 Mutation

The gain-of-function pdr9-1 mutation is highly intriguing given that it affects a domain that is extremely highly conserved in all plant PDR proteins identified to date. The proximity of this domain to the Walker motifs in the second NBD suggests that the mutation might act by increasing ATP binding or hydrolysis rates. We attempted to address this possibility using Escherichia coli expressed recombinant NBD2 in ATP hydrolysis assays as described for other ABC proteins (Jha et al., 2003). However, we could detect no activity with either the wild-type or mutant proteins.

An alternative explanation was suggested by our α-PDR9 antibody studies, in which we observed a modest (approximately 1.8-fold) but consistent increase in PDR9 protein abundance in microsomal extracts prepared from pdr9-1 roots compared to wild type. Although the precise mechanism by which the pdr9-1 mutation acts requires further study, this result suggests that the mutation may confer increased protein stability. It should be noted that several ABC transporters, including the closely related yeast PDR5p, are relatively short-lived proteins subject to ubiquitin-mediated vacuolar proteolysis (Egner et al., 1995; Egner and Kuchler, 1996). Thus, an increase in PDR9 protein levels due to enhanced stability could account for the increased 2,4-D resistance observed in pdr9-1.

Characterization of Arabidopsis Pleiotropic Drug Resistance9
Overexpression of AtPDR12 and its putative Spirodela ortholog, SpTIR2, in Arabidopsis confer increased resistance to the potential substrates lead and sciarcelor, respectively (van den Brule et al., 2002; Lee et al., 2005). We attempted to overexpress PDR9 from the cauliflower mosaic virus 35S promoter to further address this possibility but were unable to recover any transgenic lines expressing elevated levels of PDR9 (data not shown).

Regardless of the mode of action of the pdr9-1 mutation, the fact that it occurs in the extremely highly conserved PDR signature sequence suggests that the gain-of-function affects might be transferable to other plant PDR transporters. If so, this could provide a novel means for engineering plants with increased xenobiotic resistance. Such an approach may be particularly effective if transporter activity can be increased to an even greater extent by more dramatic mutations of the PDR signature motif and/or in conjunction with overexpression.

Recently, the ABC transporter encoded by the AtWBC19 gene was shown to confer kanamycin resistance when overexpressed in plants (Mentewab and Stewart, 2005). The authors noted that the development of AtWBC19 as a selectable marker provides an alternative to the bacterial-derived nptII gene was shown to confer kanamycin resistance genes. The dominant nature of the pdr9-1 mutation suggests the potential for PDR9 to be similarly developed as a plant-derived selectable marker for 2,4-D resistance, as well as the development of crops resistant to the phenoxyalkanoic acid class of herbicides, yet which exhibit normal sensitivity to endogenous auxin. Little is known regarding the function of the vast majority of plant ABC transporters. As more is learned regarding the natural substrates of these transporters as well as their capacity to mobilize various xenobiotics, this family of proteins may develop into an important genetic resource for engineering plants with increased resistance to contaminated soils and potentially for the development of phytoremediation strategies.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (Arabidopsis thaliana) lines used in this study are in the Col ecotype. Seedlings were grown under sterile conditions on ATS nutrient medium (Lincoln et al., 1990) under long-day lighting. Conditions for the mutagenesis and screen for eta 1 mutations have been previously described (Gray et al., 2003). Chemicals used in root growth assays were purchased from Sigma-Aldrich and dissolved in ethanol or dimethylsulfoxide as 1,000 ppm stocks. For root growth assays, seedlings were grown on ATS nutrient medium (Lincoln et al., 1990) under long-day lighting. Conditions for the mutation were PDR9-F4: GCGAAACTCAGAGCTTGTGA; PDR9-R1: AATGATGATTACACCGGCTCTGCCTCAAA.

GUS Histochemical Staining

A 2.3 kb fragment containing genomic sequence from upstream of the PDR9 locus through the first 33 bp of coding sequence was cloned in frame with the GUS coding sequence of pBI101.2 (CLONTECH). Seedlings were stained for GUS activity as previously described (Stomp, 1991).

Antibodies

A cDNA fragment encoding PDR9 amino acids 1 to 98 was cloned as an EcoRI-Xhol fragment into the plasmid Escherichia coli expression vector (Novagen). Expression was induced with isopropylthio-β-galactoside and the fusion protein purified on nickel-nitrilotriacetic acid agarose using standard protocols (Gray et al., 1999). The recombinant protein was eluted with imidazole and used to immunize a New Zealand white rabbit (Cocalico Biological). SEC12 and PGP4 antibodies were kindly provided by Drs. Tony Sanderfoot (University of Minnesota) and Angus Murphy (Purdue University).

Microsomal Purification and Immunoblot Analysis

Five hundred milligrams of root tissue from 8-d-old seedlings were homogenized on ice in 2 mL of buffer (250 mM sorbitol, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 7 g/L polyvinylpyrrolidone, 5 mM dithiothreitol, 0.5% protease inhibitor mix [Calbiochemistry], and 1 mM phenylmethylsulfonyl fluoride; Stukkens et al., 2005). The homogenate was centrifuged for 5 min at 10,000g at 4°C and the supernatant centrifuged again under the same conditions to remove cell debris. The resulting supernatant was spun 1.5 h at 20,000g at 4°C, and the final pellet suspended in 100 μL resuspension buffer (5 mM potassium phosphate buffer, pH 7.8, 330 mM Suc, 3 mM KCl, 0.5% proteinase inhibitor mix, and 1 mM phenylmethylsulfonyl fluoride). For the aqueous two-phase partitioning experiments, microsomes were prepared from 10-d-old roots (5 g fresh weight) and fractionated according to Larsson et al. (1994). For immunoblotting, 5 μg of each protein sample solubilized for 15 min at 37°C in SDS sample buffer were subjected to SDS-PAGE (7.5% polyacrylamide) and transferred electrophoretically to nitrocellulose membranes (Amersham). Immunoblot procedures have been described previously (Gray et al., 1999). The antibody dilutions were 1:4,000 for α-PDR9, 1:4,000 for α-ASEC12, and 1:5,000 for α-PGP4 antisera. Where indicated, quantification of immunoblots was performed using ImageJ with enhanced chemiluminescence exposures on prelabeled autoradiography film.

Labeling Assays

Root tips (apical ~ 5 mm) were excised from 5-d-old Arabidopsis seedlings and preincubated in uptake buffer (20 mM MES-KOH, pH 5.6, 10 mM Suc, 0.5 mM calcium sulfate). Then, tips were incubated in uptake buffer containing 250 mM [3H]-2,4-D, 250 mM [3H]-IAA, or 34 mM [3H]-NPA, respectively. After
60 min incubation, root tips were rinsed with same buffer and placed directly into liquid scintillation fluid. [5-3H]-IAA (20 Ci/mmol), [ring-14C (U)]-2,4-D (80 mCi/mmol), and [2,3,4,5-3H]-NPA (58 Ci/mmol) were obtained from American Radiolabeled Chemicals.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number BK001008 (PDR9).

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