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Circadian Clock-Regulated Phosphate Transporter PHT4;1 Plays an Important Role in *Arabidopsis* Defense

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ABSTRACT The *Arabidopsis accelerated cell death 6-1* (*acd6-1*) mutant shows constitutive defense, cell death, and extreme dwarf phenotypes. In a screen for *acd6-1 suppressor*s, we identified a mutant that was disrupted by a T-DNA in the *PHOSPHATE TRANSPORTER 4;1* (*PHT4;1*) gene. The suppressor mutant *pht4;1-1* is dominant, expresses truncated *PHT4;1* transcripts, and is more susceptible to virulent *Pseudomonas syringae* strains but not to several avirulent strains. Treatment with a salicylic acid (SA) agonist induced a similar level of resistance in Col-0 and *pht4;1-1*, suggesting that *PHT4;1* acts upstream of the SA pathway. Genetic analysis further indicates that *PHT4;1* contributes to *SID2*-dependent and -independent pathways. Transgenic expression of the DNA fragment containing the *PHT4;1-1* region or the full-length *PHT4;1* gene in wild-type conferred enhanced susceptibility to *Pseudomonas* infection. Interestingly, expression of *PHT4;1* is regulated by the circadian clock. Together, these data suggest that the phosphate transporter PHT4;1 is critical for basal defense and also implicate a potential role of the circadian clock in regulating innate immunity of *Arabidopsis*.

Key words: Biological clock; disease resistance; signal transduction; *Pseudomonas syringae*; phosphate transporter; salicylic acid.

INTRODUCTION

Successful control of plant diseases depends on a thorough understanding of the mechanism of disease resistance in plants. In response to pathogen attacks, plants actively reprogram expression of thousands of genes (Maleck et al., 2000; Tao et al., 2003; Katagiri, 2004), among which only a few are known to play a direct role in regulating plant defense while most of them are diagnostic of defense responses. Thus, the major challenge in the field remains to identify components in the defense signaling networks and to understand their functions in regulating disease resistance.

One of the key nodes in the defense signaling networks is centered on the small phenolic compound salicylic acid (SA). SA is required for establishment of basal defense induced by pathogen elicitors, strong local resistance in the infected region induced by pathogen effector proteins as well as systemic acquired resistance (SAR) at the whole plant level (Hammond-Kosack and Jones, 1996; Ryals et al., 1996; Tsuda et al., 2008). Several genes that are important for SA-mediated defense have been identified in *Arabidopsis* and they can be grouped into three interconnected subgroups. The type I SA regulatory genes include *SA INDUCTION-DEFICIENT 2* (*SID2*), encoding isochorismate synthase contributing to the bulk SA biosynthesis (Wildermuth et al., 2001). The type II SA regulatory genes are generally not considered to directly participate in SA biosynthesis because the protein products of these genes lack distinct enzymatic motifs. Examples of the type II SA regulators include *ACCELERATED CELL DEATH 6* (*ACD6*), *AGD2-LIKE DEFENSE 1* (*ALD1*), *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *PHYTOALEXIN DEFICIENT 4*, *(PAD4) SID1/EDS5*, *HOPW1-1-INTERACTING/AVRPPHB SUSCEPTIBLE/GH3-LIKE DEFENSE GENE 1*, and the *MODIFIER OF SNC1* genes (Falk et al., 1999; Jirage et al., 1999; Nawrath et al., 2002; Lu et al., 2003; Song et al., 2004; Palma et al., 2005; Zhang et al., 2005; Zhang and Li, 2005; Goritschnig et al., 2007; Jagadeeswaran et al., 2007; Lee et al., 2007b; Nobuta et al., 2007; Palma et al., 2007). However, how some of these genes influence SA accumulation still remains to be determined (Lu, 2009). Genes acting downstream of SA signaling comprise the type III SA regulatory genes. The best-characterized defense

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gene in this group is *NONEXPRESSOR OF PR GENES 1* (*NPR1*), the protein product of which translocates from the cytoplasm to the nucleus in response to redox changes to control defense gene expression and SAR activation (Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997; Mou et al., 2003; Dong, 2004; Tada et al., 2008). To increase the complexity of defense signaling networks, SA is also known to cross-talk with signals derived from several phytohormones (Feys and Parker, 2000; Kunkel and Brooks, 2002; Wang et al., 2007; Koornneef and Pieterse, 2008; de Torres Zabala et al., 2009).

The type II SA regulator ACD6 is an ankyrin-repeat protein with a transmembrane domain and was recently shown to be a major determinant of fitness in *Arabidopsis* (Todesco et al., 2010). Loss-of-function mutation in the *ACD6* gene leads to reduced SA accumulation and compromised defense against *Pseudomonas syringae* infection. In contrast, a gain-of-function mutant, *acd6-1*, caused by one amino acid substitution in the transmembrane domain of ACD6, exhibits extreme dwarfism and constitutive resistance to broad-spectrum pathogens, including *P. syringae*, *Hyaloperonospora arabidopsidis*, and *Botrytis cinerea* (Rate et al., 1999; Lu et al., 2003; Song et al., 2004; Wang and Lu, unpublished data). *acd6-1* also accumulates high levels of SA and camalexin (an anti-fungal metabolite) and displays severe cell death. Interestingly, the small size of *acd6-1* inversely correlates with the defense levels in the plant (Song et al., 2004; Lu et al., 2009). We took advantage of this unique feature of *acd6-1* in a mutant screen for *acd6-1 suppressors* (*sups*), which are larger plants with potential disruptions in novel defense genes (Lu et al., 2009). T-DNA mutagenesis was used to introduce second site mutations in the *acd6-1* background and to facilitate the subsequent cloning of the disrupted gene. Among 30 *sup* mutants isolated, we identified an allele of *SID2* and cloned the *SUP6* gene, encoding a predicted transmembrane protein with an N-terminal peptidase domain (Lu et al., 2009). Therefore, we have validated that *acd6-1* suppressor screen is powerful in uncovering novel genes important for defense responses.

In this study, we report the isolation and characterization of a suppressor mutant that harbors a T-DNA insertion in the *PHOSPHATE TRANSPORTER 4;1* (*PHT4;1*) gene (Guo et al., 2008a, 2008b). *PHT4;1*, also named *ANTR1* (Roth et al., 2004; Pavon et al., 2008), belongs to a six-gene family in *Arabidopsis*. Only *PHT4;6* was shown to regulate plant response to salt stress (Cubero et al., 2009); the biological functions of other members in the *PHT4* family are largely unknown. We showed here that the suppressor mutant *pht4;1-1* expressed truncated *PHT4;1* transcripts and was dominant. *pht4;1-1* conferred enhanced disease susceptibility to virulent *Pseudomonas* strains and this susceptibility could be suppressed by the treatment of an SA agonist. In addition, we showed that transgenic Col-0 plants carrying one or more copies of the truncated *PHT4;1-1* genomic fragment or the full-length *PHT4;1* gene were more susceptible to *Pseudomonas* infection. Thus, we provided the first evidence to implicate a member in the *PHT4* family in regulating plant innate immunity. Interestingly, we found that expression of *PHT4;1* was regulated by the biological clock, suggesting a role for the biological clock in control of disease resistance in plants.

RESULTS

pht4;1-1 Suppresses *acd6-1*-Conferred Phenotypes

We previously showed that the small size of *acd6-1* is grossly in inverse correlation with defense levels in the plant. We took advantage of this unique feature of *acd6-1* in a suppressor screen in order to discover novel defense genes. Among the *suppressor* (*sup*) mutants isolated, *acd6-1sup3-1*, later designated *acd6-1pht4;1-1*, has an intermediate size compared with *acd6-1* and Col-0 (Figure 1A). The size phenotype of *acd6-1pht4;1-1* was confirmed in progenies of two backcrosses. Consistent with the change in plant size, *pht4;1-1* partially suppressed *acd6-1* for the expression of the defense marker gene *PATHOGENESIS RELATED 1* (*PR1*) and SA accumulation (Glazebrook et al., 1997) (Figure 1B and 1C). When challenged with the virulent bacterium, *Pseudomonas syringae* pv. *maculicola* strain DG3 (*Pma*DG3), *pht4;1-1* partially suppressed constitutive defense in *acd6-1* (Figure 1D).

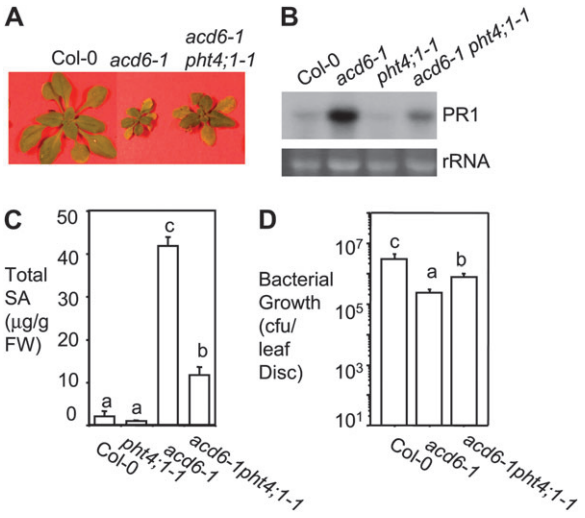

Figure 1. The *pht4;1-1* Mutant Suppresses *acd6-1*-Conferred Phenotypes.

(A) Picture of 25-day-old plants.

(B) Northern blot analysis of *PR1* expression. Total RNA was isolated from 25-day-old uninfected plants. *rRNA* was used as a loading control.

(C) SA quantification. Uninfected 25-day-old plants were harvested for SA extraction followed by HPLC analysis.

(D) Bacterial growth assay. 25-day-old plants were infected with *Pseudomonas syringae* pv. *maculicola* strain DG3 (*Pma*DG3) (OD_{600} = 0.0001) and leaf discs were collected for bacterial growth assay 3 d after infection. CFU, colony forming unit.

Different letters in (C) and (D) indicate significant difference among samples ($P < 0.05$; $n = 3$ in (C) and $n = 6$ in (D)). These experiments were repeated two times, with similar results.

pht4;1-1 Is Dominant and Confers Enhanced Disease Susceptibility to Virulent *Pseudomonas* Strains

To further investigate the role of *pht4;1-1* in defense regulation, we crossed *acd6-1pht4;1-1* with Col-0 and obtained the *pht4;1-1* homozygous mutant in the absence of *acd6-1*. We challenged *pht4;1-1* and Col-0 with both virulent and avirulent *Pseudomonas* strains. We found that *pht4;1-1* showed significantly more growth of two virulent strains (*Pma*DG3 and *P. syringae* pv. *tomato* strain DC3000 (DC3000)), compared to Col-0 (Figure 2A and 2B). *pht4;1-1* also showed more severe disease symptoms than Col-0 with *Pma*DG3 infection (Supplemental Figure S1). However, similar susceptibility was found in *pht4;1-1* and Col-0 to the avirulent strains, *Pma* avrRpt2 or *Pma* avrRpm1 (Figure 2C). These data suggest that *PHT4;1* is involved in basal defense but not in defense mediated by R genes, such as *RPS2* and *RPM1*. Surprisingly, we found a single copy of the *pht4;1-1* mutation conferred enhanced disease susceptibility to *PmaDG3* (Figure 2D), suggesting a dominant nature of the *pht4;1-1* mutation.

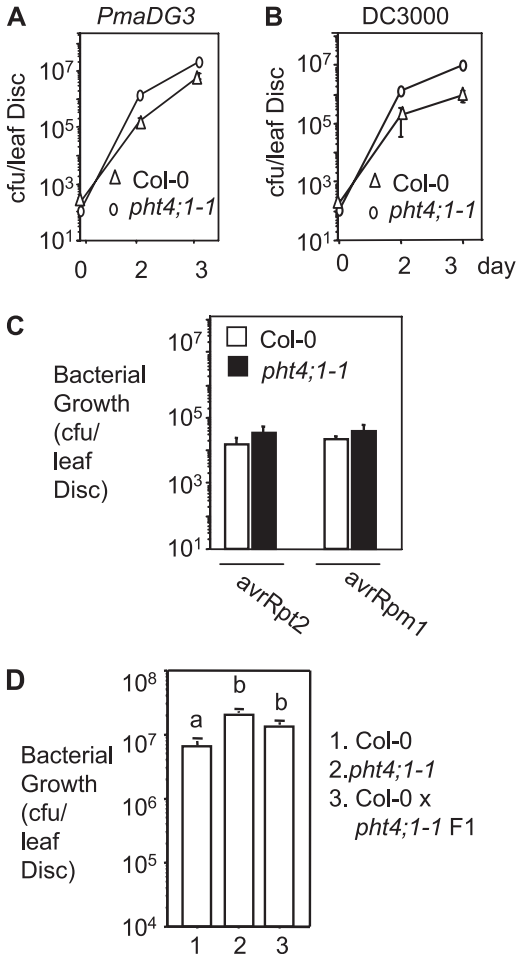

Figure 2. *pht4;1-1* Is Dominant and Confers Enhanced Disease Susceptibility to Virulent *Pseudomonas* Strains.

(A) Infection with *Pma*DG3 (OD_{600} = 0.0001).

(B) Infection with *P. syringae* pv. *tomato* DC3000 (DC3000) (OD_{600} = 0.0001).

(C) Infection with *Pma avrRpt2* or *Pma avrRpm1* (OD_{600} = 0.0002).

(D) Infection of heterozygous *pht4;1-1* with *Pma*DG3 (OD_{600} = 0.0001).

25-day-old plants were infected with the indicated *Pseudomonas* strains and assayed for bacterial growth. Significant difference between *pht4;1-1* and Col-0 was observed at 2 and 3 days after infection in (A) and (B) and was indicated with different letters in (D) ($P < 0.05$; $n = 6$). No difference was observed in the two samples in (C) 3 d after infection with either avirulent strain. These experiments were repeated two times, with similar results.

PHT4;1 Encodes a Phosphate Transporter

Using the TAIL–PCR method (Liu et al., 1995), we identified a T-DNA insertion in the fifth exon of the *PHT4;1* gene (At2g29650) in the *pht4;1-1* mutant (Figure 3A). Instead of abolishing gene expression, the T-DNA insertion led to the

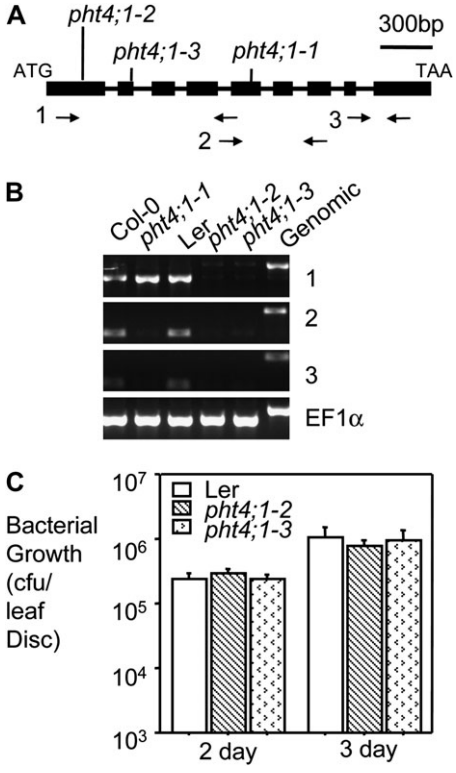

Figure 3. Two *PHT4;1* Null Alleles Are Not Compromised in Disease Resistance.

(A) Structure of the *PHT4;1* gene and positions of the mutant alleles. Filled boxes indicate exons and horizontal lines indicate introns. Vertical lines indicate the positions of *PHT4;1* alleles. The arrow pairs are primer sets used in RT–PCR in (B).

(B) RT–PCR analysis. EF1α was used as a loading control. Note the PCR products amplified from a genomic DNA template included introns and therefore were larger than their corresponding RT–PCR products.

(C) Bacterial growth assay. 25-day-old plants were infected with *Pma*DG3 (OD_{600} = 0.0002). Six leaf discs from infected individual plants of each genotype were collected for bacterial growth assay 3 d after infection. No significant difference was observed among the genotypes. Experiments in (B) and (C) were repeated three times, with similar results.

production of a truncated transcript from the 5′ end of this gene (Figure 3B), which presumably encodes a truncated PHT4;1 protein with the first 347 amino acids. *PHT4;1*, previously also named *ANTR1* (Roth et al., 2004; Guo et al., 2008b; Pavon et al., 2008), encodes a transmembrane protein that belongs to a small family with six members. Recently, members of the *PHT4* family were demonstrated to have phosphate uptake activity (Guo et al., 2008b; Pavon et al., 2008; Cubero et al., 2009). One of the *PHT4* family members (*PHT4;6*) was shown to regulate plant response to salt stress (Cubero et al., 2009). However, the biological functions of *PHT4;1* and other members in the family are largely unknown.

We identified two loss-of-function alleles of *PHT4;1* in Landsberg accession—*pht4;1-2* (GT_5_110509) and *pht4;1-3* (CSHL_GT22119)—both of which were disrupted by transposon insertions (Figure 3A). RT–PCR analysis indicated that *PHT4;1* expression was completely abolished in these two alleles (Figure 3B), suggesting that they are null mutants. However, unlike *pht4;1-1*, *pht4;1-2* and *pht4;1-3* showed similar disease resistance to *Pseudomonas* as wild-type (WT) (Figure 3C). Since there are five other *PHT4;1*-like genes in *Arabidopsis*, it is possible that some of these genes share redundant function with *PHT4;1*.

To confirm that *pht4;1-1*-conferred phenotypes are due to the perturbation of the *PHT4;1* gene, we made transgenic Col-0 plants carrying one or more copies of the genomic fragment of *PHT4;1-1* (encoding the N-terminal 1–347 amino acid of the protein) driven by the native *PHT4;1* promoter. Like *pht4;1-1*, most transgenic plants were more susceptible than the control to *Pma*DG3 infection. In addition, we generated transgenic Col-0 plants expressing extra copies of the full-length *PHT4;1* gene. Five out of seven such transgenic plants showed increased susceptibility to *Pma*DG3 infection. Bacterial growth results for two representative lines from each transformation are shown in Figure 4. Together, these data indicate that T-DNA disruption of the *PHT4;1* gene is responsible for the *pht4;1-1*-conferred phenotypes and that *PHT4;1* is a negative regulator of plant defense.

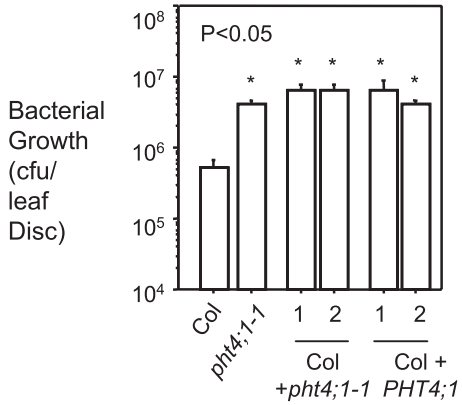

Figure 4. Transgenic Plants Expressing *PHT4;1-1* or *PHT4;1* Are More Susceptible to *Pma*DG3 Infection.

A genomic fragment containing the *PHT4;1-1* region or the full-length *PHT4;1* gene was PCR amplified and cloned into the binary vector pGreenII 0029 (Hellens et al., 2000) for plant transformation. The *PHT4;1* promoter (1329 bp) was used to drive the expression of these two constructs. 25-day-old plants were infected with *Pma*DG3 (OD_{600} = 0.0001) and leaf discs were collected for bacterial growth assay 3 d after infection. Asterisks indicate significant difference between Col-0 and the transgenic plants ($P < 0.05$; $n = 6$). These experiments were repeated two times, with similar results.

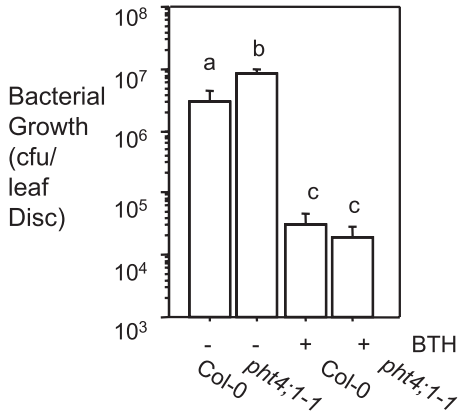

Figure 5. *pht4;1-1*-Conferred Susceptibility Can Be Suppressed by BTH Treatment.

Plants were treated with 300 μM BTH for 36 h before *Pma*DG3 infection (OD_{600} = 0.0001). Different letters indicate significant difference among samples ($P < 0.05$; $n = 6$). These experiments were repeated two times, with similar results.

pht4;1-1 Contributes to SA-Mediated Defense

SA plays a key role in signaling plant defense (Hammond-Kosack and Jones, 1996; Ryals et al., 1996; Tsuda et al., 2008). To test whether *pht4;1-1*-conferred susceptibility is SA-related, we pre-treated Col-0 and *pht4;1-1* with 300 μM benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH), an agonist of SA (Lawton et al., 1996), and subsequently challenged the plants with *Pma*DG3. We found that BTH pretreatment induced a similar level of resistance in Col-0 and *pht4;1-1* (Figure 5), suggesting that the *pht4;1-1* mutation does not impair the ability of the plant to induce SA-mediated defense in response to exogenous BTH. Thus, *PHT4;1* likely acts upstream of SA signaling.

To further investigate how *PHT4;1* affects SA-mediated defense, we crossed *acd6-1pht4;1-1* to *acd6-1sid2-1*. *SID2* is known to contribute to bulk SA biosynthesis and the *sid2-1* mutation abolishes most SA accumulation in *acd6-1* (Lu et al., 2009). The fact that *acd6-1pht4;1-1* accumulated intermediated SA levels compared with Col-0 and *acd6-1* (Figure 1C) suggests that *pht4;1-1* partially affects *SID2*-mediated SA biosynthesis. If *pht4;1-1* also impairs *SID2*-independent SA biosynthesis, we expect that the triple mutant *acd6-1pht4;1-1sid2-1* would be larger than the parental double mutants. Otherwise, if *pht4;1-1* impairs only *SID2*-dependent SA biosynthesis, the triple mutant should be similar to the two parental double

mutants. We found that the former case was true for *acd6-1pht4;1-1sid2-1* (Figure 6A).

acd6-1 exhibited severe cell death, which could be suppressed by the *sid2-1* mutation (Figure 6B; Lu et al., 2009). We found that the *pht4;1-1* mutant also suppressed the severity of cell death in *acd6-1*. In addition, consistent with its increased size, *acd6-1pht4;1-1sid2-1* had much reduced cell death compared with *acd6-1pht4;1-1* and *acd6-1sid2-1* (Figure 6B). Furthermore, we found that *acd6-1pht4;1-1sid2-1* accumulated lower total SA (0.07 ± 0.05 μg gFW^{-1}) than *acd6-1pht4;1-1* (5.73 ± 0.64 μg gFW^{-1}) and *acd6-1sid2-1* (0.22 ± 0.14 μg gFW^{-1}). Since the protein product of *PHT4;1* lacks distinct motifs for being SA biosynthetic enzyme, we concluded from our studies that *PHT4;1* is a type II SA regulator contributing to both *SID2*-dependent and -independent SA accumulation.

Consistent with *pht4;1-1* being dominant (Figure 2D), we found that, among 114 progenies of a heterozygous triple mutant, *acd6-1/acd6-1;sid2-1/sid2-1;pht4;1-1/PHT4;1*, 33 plants were smaller in size, while 81 plants were larger (a 1:3 ratio; $X^2 = 0.45$; $P > 0.5$). The smaller plants were genotyped to be *acd6-1sid2-1* homozygous, had no *pht4;1-1* allele, and were BASTA-sensitive. The larger ones were indistinguishable in size and were BASTA-resistant (Figure 6A). They segregated into 24 homozygous triple mutants and 57 heterozygotes (*acd6-1/acd6-1;sid2-1/sid2-1;pht4;1-1/PHT4;1*). These observations indicate that *pht4;1-1* is due to a single T-DNA insertion, which is associated with the increased size phenotype and BASTA resistance. In addition, the fact that disrupting one copy of *PHT4;1-1* is sufficient to suppress *acd6-1sid2-1* phenotypes further supports that the *pht4;1-1* mutation is dominant.

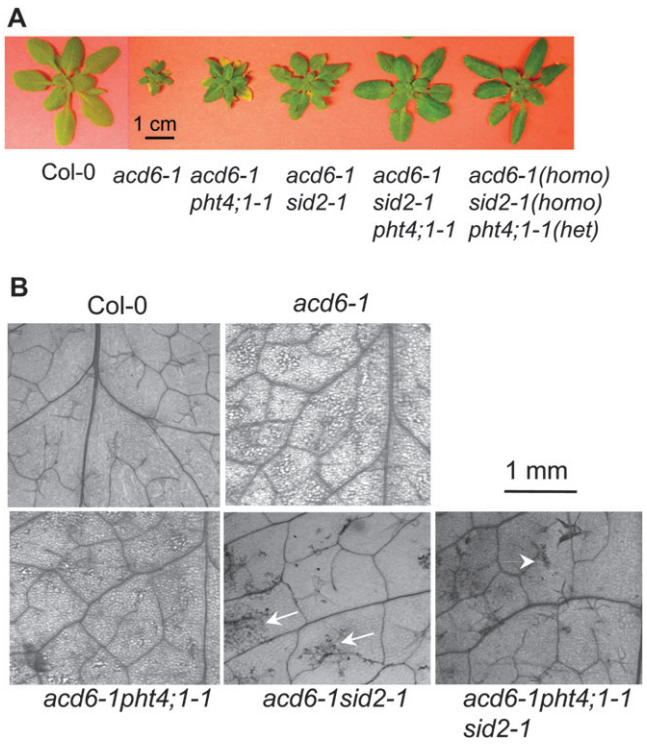

Figure 6. The *pht4;1-1* Mutation Contributes to *SID2*-Dependent and -Independent Pathways.

(A) Picture of 25-day-old plants. Note the triple mutants are larger than the double mutants.

(B) Cell death phenotypes. The fifth leaves of the indicated genotypes were stained with trypan blue and photographed with a Photometrics CCD camera connected to a Leica dissecting microscope. Arrows indicate minor cell death in *acd6-1sid2-1* and *acd6-1pht4;1-1sid2-1*. These experiments were repeated two times, with similar results.

PHT4;1 Expression Is Regulated by Light and the Circadian Clock

Bioinformatics analysis of publicly available microarray database (Genevesitgator; (Zimmermann et al., 2004)) and a previous study from Guo et al. (2008a) suggest that light regulates *PHT4;1*. To further investigate the role of light in regulating *PHT4;1* expression, we performed northern blotting with total RNA extracted from Col-0, *acd6-1*, *pht4;1-1*, and *acd6-1pht4;1-1* in the presence or absence of light. Figure 7 shows that 24 h dark treatment completely abolished expression of *PHT4;1* in Col-0 and *acd6-1* and the truncated version *PHT4;1-1* in the *pht4;1-1* background. These observations suggest that light is required for *PHT4;1* expression while dark suppresses its expression.

The circadian clock was also shown to regulate *PHT4;1* expression (Guo et al., 2008a). In particular, two copies of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)-binding site (CBS) were found in the *PHT4;1* promoter. CBS is a *cis*-element important for morning-specific circadian expression regulated by the central oscillator component of the circadian clock, CCA1 (Wang et al., 1997; Michael and McClung, 2002). To further investigate circadian regulation of *PHT4;1* expression, we harvested leaf tissues from Col-0 and *pht4;1-1* at every 4-h interval in 32 h under a light/dark cycle. RNA analysis revealed that both *PHT4;1* and the truncated version *PHT4;1-1* exhibited a similar diurnal expression pattern, with peaks in the daytime and troughs in the nighttime (Supplemental Figure S2). Such an expression pattern of *PHT4;1* was

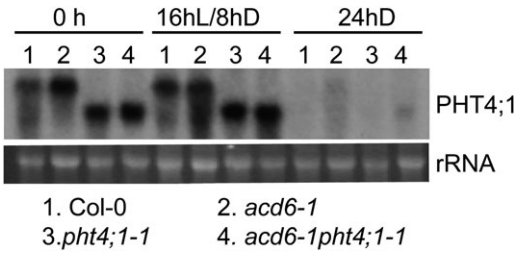

Figure 7. Expression of *PHT4;1* and *PHT4;1-1* Is Light-Regulated.

25-day-old plants grown in a chamber with a 16-h light/8-h dark cycle were kept in the same condition or dark-treated for 24 h. Leaves were harvested for total RNA extraction followed by northern blotting. *rRNA* was used as a loading control. These experiments were repeated two times, with similar results.

also observed in the *acd6-1* background under a light/dark cycle and persisted under a continuous light condition (Figure 8A). In contrast, expression of *NPR1* was constant in *acd6-1*. In addition, we found that the diurnal expression of *PHT4;1* was unaffected by *Pma*DG3 infection, although a slight suppression of *PHT4;1* abundance was observed at 16 h and 20 h after *Pma*DG3 infection. As a control, the level of *NPR1* transcripts was induced 12 h after the infection (Figure 8B). These observations strongly suggest that expression of *PHT4;1* is under the control of the circadian clock.

DISCUSSION

From the *acd6-1* suppressor screen, we isolated a mutant that harbors a T-DNA insertion in *PHT4;1*, the protein product of which was demonstrated to have phosphate transport activity (Roth et al., 2004; Guo et al., 2008b; Pavon et al., 2008). *PHT4;1*, also named *ANTR1*, belongs to a small gene family with six members, functions of which are largely unknown. In this study, we provided evidence to implicate a role of *PHT4;1* in regulating plant defense. We showed that *pht4;1-1* was dominant, possibly due to expression of the truncated *PHT4;1*. *pht4;1-1* suppresses *acd6-1*-conferred phenotypes and is more susceptible to virulent *Pseudomonas* strains but not to two avirulent strains. The *pht4;1-1*-conferred susceptibility could be suppressed by treatment with BTH, an SA agonist and recapitulated by transgenically expressing a *PHT4;1-1* or *PHT4;1* genomic fragment in Col-0. Together, our data suggest that PHT4;1 is a negative regulator of basal defense in *Arabidopsis*. Interestingly, expression of *PHT4;1* was under circadian control, suggesting a potential role of the biological clock in regulating plant innate immunity.

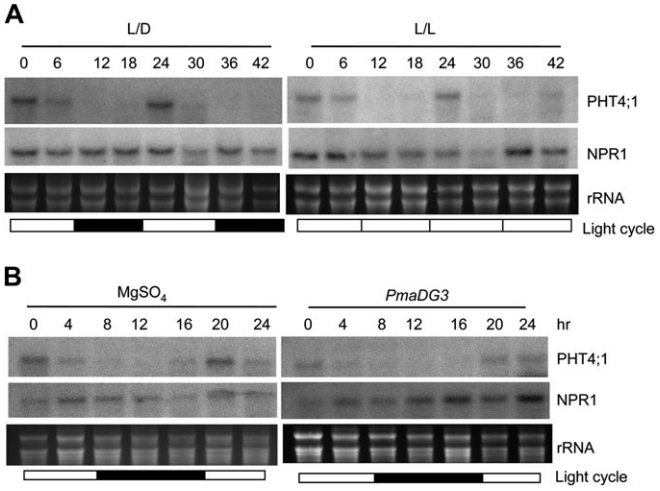

Figure 8. Expression of *PHT4;1* Is Regulated by the Circadian Clock.
(A) Circadian expression of *PHT4;1* in *acd6-1*. 25-day-old *acd6-1* plants grown in a chamber with a 12-h light/12-h dark cycle were kept in the same chamber or moved to a chamber with continuous light conditions. Starting from time 0 (9:00 am), plants were harvested every 6 h for 42 h.
(B) Circadian expression of *PHT4;1* during *Pseudomonas* infection. 25-day-old Col-0 plants grown in a chamber with a 12-h light/12-h dark cycle were infiltrated with *Pma*DG3 (OD = 0.0001) or 10 mM $MgSO_4$ as control. The infiltrated leaves were collected at 4-h interval for 24 h.
Total RNA were extracted from the above samples and analyzed by northern blotting. White boxes indicate light periods and black boxes indicate dark periods. *rRNA* was used as a loading control. These experiments were repeated two times, with similar results.

The Role of PHT4;1-Mediated Phosphate Transport in Plant Defense

Phosphorus (P) is an essential element for all living organisms and plays a crucial role in many physiological processes. Plant cells obtain their P via absorbing inorganic phosphate (Pi; the assimilable form of P) directly from the soil and/or from neighboring cells. Intracellular Pi can also be reallocated to different organelles of the cell. These processes are mediated by Pi transporters. The PHT4 family is one of the four Pi transporter families in *Arabidopsis*. Additional non-transporter genes are also known to regulate Pi sensing and signaling, leading to altered Pi transport and subsequently affecting Pi homeostasis in plants (Poirier and Bucher, 2002; Lin et al., 2009). Although mutations in many genes are known to influence Pi homeostasis in plants, only a few of such genes are known to be involved in defense regulation (Miura et al., 2005; Lee et al., 2007a; Murphy et al., 2008), suggesting that defense activation is not due to a general perturbation of Pi homeostasis in the cell.

pht4;1-1 and its two loss-of-function alleles are not known to have altered responses to Pi starvation (Lu, unpublished data and Wayne Versaw, personal communication), suggesting that PHT4;1 does not contribute to major Pi homeostasis in the cell. Evidence from our studies implicates a role of PHT4;1 in regulating plant defense, possibly by acting upstream of SA. Thus, we hypothesize that, rather than disrupting the Pi homeostasis in the cell, Pi reallocation mediated by PHT4;1 may generate a signal to regulate SA-mediated plant defense. A candidate downstream signaling could be derived from the second messenger molecule inositol (1, 4, 5) phosphate (InsP3), which is involved in regulating both biotic and abiotic stress responses in plants. InsP3 is generated by phospholipase C (PLC) catalyzing the hydrolysis of phosphatidylinositol-4,5-bisphosphate, a process activated by biotic and abiotic stimuli (Mueller-Roeber and Pical, 2002). Inositol 5-phosphatases (5PTases) terminate InsP3 signaling by hydrolyzing InsP3 (Erneux et al., 1998). One previous study showed that expression of *PHT4;1* is co-regulated with InsP3 accumulation in the *5ptase* mutant (Chen et al., 2008). This observation suggests a possibility that Pi transport by PHT4;1 is important for InsP3 signaling, which subsequently leads to defense responses. Further studies from metabolite analysis and genetics should reveal whether the *pht4;1-1* mutant affects IP3 levels and, if so, how PHT4;1-mediated IP3 signaling is involved in regulating plant disease resistance.

Functional Redundancy among Members in the PHT4 Family

We showed that extra copies of *PHT4;1* confer enhanced disease susceptibility, suggesting that PHT4;1 is a negative regulator of plant defense. However, we did not observe altered defense phenotypes in the two loss-of-function alleles (Figure 3). This is probably due to functional redundancy between PHT4;1 and other members in the family. Indeed, PHT4;1 shares high homology (from 27 to 65% identity) with five other family members. Except PHT4;6, all other members were localized or predicted to be in the plastid (Roth et al., 2004; Guo et al., 2008a; Pavon et al., 2008; Cubero et al., 2009). Thus, it is tempting to speculate that, like PHT4;1, some members in the PHT4;1 family also play a role in regulating plant defense. Further mutant analysis with multiple PHT4 family members being disrupted should reveal this possibility.

Bioinformatics analysis also revealed that PHT4;1-like proteins are present in diverse organisms, including plants and animals. Interestingly, a high degree of conservation (about 30%) is also shared between PHT4;1 and proteins from evolutionarily distant organisms, such as human sialin, rat VGLUT1, and VGLUT2 proteins, mouse Npt1, rabbit NaPi-1, and EAT-4 of *Caenorhabditis elegans* (Werner et al., 1991; Ni et al., 1994; Chong et al., 1995; Lee et al., 1999; Verheijen et al., 1999; Aihara et al., 2000). Notably, mutations in VGLUT1, VGLUT2, and EAT-4 result in severe neuronal diseases associated with aberrant cell death in rat and *C. elegans* (Raizen and Avery, 1994; Verheijen et al., 1999; Wojcik et al., 2004; Tordera et al., 2007; Garcia-Garcia et al., 2009). Consistent with the function of its animal homologs, we show here that PHT4;1 regulates disease resistance and cell death in *Arabidopsis*. Like some animal homologs, PHT4;1 family members were also demonstrated to have Pi transport activities (Guo et al., 2008a; Pavon et al., 2008; Cubero et al., 2009). Together, these observations suggest a common mechanism for the action of PHT4;1 and its homologs from plants and animals.

Like introducing extra copies of PHT4;1, the *pht4;1-1* mutation also confers enhanced disease susceptibility, suggesting that the predicted PHT4;1-1 protein is gain-of-function in nature. For instance, the PHT4;1-1 protein might have an altered biochemical activity by changing the kinetics of substrate uptake and/or transporting Pi in a different direction at the organelle level, compared with the WT protein. It is also possible that PHT4;1-1 might be permeable to a different substrate profile compared with the WT protein. Further elucidation of the biochemical activity of PHT4;1 and PHT4;1-1 should facilitate the understanding of mechanisms of action of these proteins in plant defense and also reveal whether *pht4;1-1* is a neomorphic or hypermorphic mutation.

A Potential Role of the Circadian Clock in Regulating Plant Defense

The circadian clock is the intrinsic time-measuring machinery that plays a central role in regulating plant growth and development and responses to environmental stimuli, such as light. Although light has long been implicated in regulating plant innate immunity (for review, see Roden and Ingle, 2009), whether or not the circadian clock regulates plant defense has not been well understood. However, previous studies that showed expression of some pathogen-inducible genes oscillated in a circadian manner indeed suggest such a possibility (Wang et al., 2001; Sauerbrunn and Schlaich, 2004; Weyman et al., 2006). To further corroborate this notion, we show here that expression of *PHT4;1* is regulated by both the circadian clock and light (this study and Guo et al., 2008a). *PHT4;1* is likely a target of the central oscillator component CCA1, since two copies of CBS were identified in the *PHT4;1* promoter. CBS was also identified in the promoters of several key SA regulatory genes, such as *SID2*, suggesting a potential role of CCA1 in defense regulation. Further genetic and molecular analysis should reveal whether CCA1 regulates expression of *PHT4;1* and other defense regulators and subsequently leads to altered defense responses.

METHODS

Plant Growth and Mutant Isolation

Unless otherwise indicated, all plants used in this paper are in Columbia background. Plants were grown in growth chambers with 200 μmol m^{-2} s^{-1} light intensity, 16-h light/8-h dark cycle, and 60% humidity. The mutants, *acd6-1* and *acd6-1sid2-1*, and *acd6-1* suppressor screen were described before (Lu et al., 2009). The *acd6-1pht4;1-1sid2-1* triple mutant was made by crossing *acd6-1pht4;1-1* and *acd6-1sid2-1* and confirmed with a cleaved amplified polymorphic sequence (dCAPS) marker for *acd6-1* and *sid2-1* (Lu et al., 2009) and PCR markers to detect T-DNA insertion in *pht4;1-1*. *pht4;1-2* (GT_5_110509) and *pht4;1-3* (CSHL_GT22119), both disrupted by a transposon tag, were in Landsberg accession and were obtained, respectively, from Arabidopsis Biological Resource Center (ABRC) at Ohio State University and from the Martienssen's Laboratory at Cold Spring Harbor Laboratory in New York. Both mutants were confirmed with corresponding PCR markers for the DNA insertions. Primers used to detect *pht4;1* mutants are listed in Supplemental Table S1.

Bacterial Infection

Bacterial strains *Pseudomonas syringae* pv. *maculicola* (*Pma*) ES4326 strain DG3 (*Pma*DG3), *Pma* avrRpt2, *Pma* avrRpm1, and *P. syringae* pv. *tomato* strain DC3000 (DC3000) were described previously (Guttman and Greenberg, 2001; Lee et al., 2007b). The fifth to seventh leaves of 25-day-old plants were infected with *Pseudomonas* strains by infiltration with a 1-ml needleless syringe. Details for bacterial culturing, infection, and growth analysis were described before (Greenberg et al., 2000; Lu et al., 2003). For benzo(1,2,3) thiadiazole-7-carbothioic acid (BTH) treatment, 25-day-old plants were sprayed

with 300 μM BTH or water for 36 h before *Pseudomonas* infection. BTH was kindly provided by Robert Dietrich (Syngenta).

SA Measurement

Total SA were extracted from 25-day-old plants as previously described (Lu et al., 2003; Song et al., 2004) and quantified with a Dionex AS50 HPLC instrument with Acclaim® 120 C18 reverse column (4.6 × 250 mm) and RF2000 fluorescence detector. O-anisic acid was used as an internal standard. SA and o-anisic acid were eluted with a gradient of methanol and 0.5% acetic acid, with o-anisic acid being detected at 4.9 min with 301-nm excitation/365-nm emission and SA being detected at 6.5 min with 301-nm excitation/412-nm emission. The final concentration of each sample was calculated based on the average of three replicates, using a standard curve made from quantification of o-anisic acid and SA at concentrations of 50, 100, 250, 500, and 1000 ng ml^{-1}.

Cell Death Staining

The fifth and sixth leaves of 25-day-old plants were collected for trypan blue staining for cell death (Rate et al., 1999). Stained leaves were examined with a Leica dissecting microscope and images were captured with a Photometrics CCD camera connected to the microscope and analyzed by MetaMorph image software.

RNA Analysis

Total RNA from 25-day-old plants was isolated using TRIzol reagent (Invitrogen). Northern blotting was performed as previously described (Lu et al., 2003). To make radioactive probes, polymerase chain reaction (PCR) was performed with primers and a DNA template specific for each gene in the presence of [^{32}P]dCTP. For reverse-transcriptase PCR (RT–PCR), total RNA was reverse-transcribed into cDNAs, using the First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. Gene-specific primers were designed to span two exons sandwiched with an intron and were used in PCR to amplify the cDNA fragment of the corresponding gene, using RT products as templates. PCR products amplified from genomic DNA from Col-0 were different in size from their corresponding RT–PCR products and were used as controls. PCR products at 25 and 30 cycles were collected and separated on a 1.5% agarose gel. Primers used to make radioactive probes and in RT–PCR are listed in Supplemental Table S1.

DNA Construction and Plant Transformation

The *PHT4;1-1* genomic DNA, including 1329-bp promoter region, was PCR amplified with At2g29650longST_493F and SUP3_T-DNA5′ primers and cloned into the binary vector pGreenII 0029 (Hellens et al., 2000). The construct was transferred to *Agrobacterium* GV3101 for plant transformation, according to the floral dipping method (Clough and Bent, 1998). The full-length *PHT4;1* genomic fragment was similarly cloned, using primers At2g29650longST_493F and At2g29650longST_4617R, and transferred to Col-0. Plant transformants were selected on soil by spraying with BASTA at a dilution of 1:4000 (AgrEvo USA, Wilmington, DE). At the T_2 generation, homozygous lines were selected on MS agar plates containing the herbicide BASTA and further planted on soil for resistance test with *Pseudomonas* infection. Primers used to make DNA constructs are listed in Supplemental Table S1.

Accession Number

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number At2g29650.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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