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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).

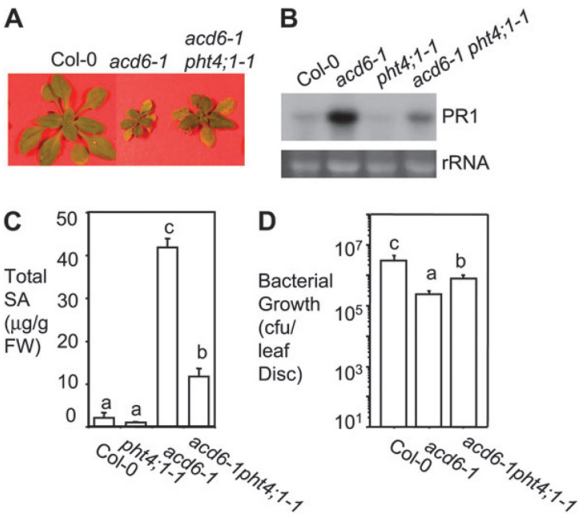

Figure 1. The *pht4;1-1* Mutant Suppresses *acd6-1*-Conferred Phenotypes.

(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.