Specific Targeting of the *Arabidopsis* Resistance Protein RPW8.2 to the Interfacial Membrane Encasing the Fungal Haustorium Renders Broad-Spectrum Resistance to Powdery Mildew

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Powdery mildew fungal pathogens penetrate the plant cell wall and develop a feeding structure called the haustorium to steal photosynthetate from the host cell. Here, we report that the broad-spectrum mildew resistance protein RPW8.2 from *Arabidopsis thaliana* is induced and specifically targeted to the extrahaustorial membrane (EHM), an enigmatic interfacial membrane believed to be derived from the host cell plasma membrane. There, RPW8.2 activates a salicylic acid (SA) signaling-dependent defense strategy that concomitantly enhances the encasement of the haustorial complex and onsite accumulation of H$_2$O$_2$, presumably for constraining the haustorium while reducing oxidative damage to the host cell. Targeting of RPW8.2 to the EHM, however, is SA independent and requires function of the actin cytoskeleton. Natural mutations that impair either defense activation or EHM targeting of RPW8.2 compromise the efficacy of RPW8.2-mediated resistance. Thus, the interception of haustoria is key for RPW8-mediated broad-spectrum mildew resistance.

**INTRODUCTION**

Plant defenses against invading obligate, biotrophic haustorium-forming fungal, and oomycete pathogens occur at preinvasin and postinvasin stages (Lipka et al., 2005). Preinvasin resistance protects plants from nonadapted pathogens by blocking their entry into the host cell (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). One common induced cellular defense response is the deposition of defense chemicals, including callose (β-1,3-glucan) at the site of penetration, resulting in cell wall apposition, a subcellular structure also known as a papilla (Aist, 1976; Huckelhoven et al., 1999). Plants have evolved at least two distinct mechanisms for induction of preinvasin resistance. The first engages the PENETRATION1 (PEN1) syntaxin-containing SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex-dependent exocytosis pathway for delivering yet-to-be-defined antimicrobial materials to the site of penetration (Collins et al., 2003; Kwon et al., 2008). The second involves the accumulation of 4-methoxyindol-3-ylmethylglucosinolate (which leads to callose formation) through hydrolysis of indole glucosinolates by the PEN2 myrosinase and action of the plasma membrane–localized PEN3 ATP binding cassette transporter (Lipka et al., 2003; Stein et al., 2006; Bednarek et al., 2009; Clay et al., 2009). Loss of function of PEN1, PEN2, or PEN3 in *Arabidopsis thaliana* each results in higher penetration rates of nonadapted powdery mildew pathogens.

For an adapted pathogen, however, preinvasin resistance is ineffective; the pathogen has evolved the ability to penetrate the host cell wall underneath the appressorium and produce a feeding structure, the haustorium, presumably through invagination of the host plasma membrane (PM). This interfacial membrane, termed extrahaustorial membrane (EHM) (Mackie et al., 1991), is poorly characterized both in terms of its lipid and protein constituents. Apart from nutrient uptake from the host cell (Manners and Gay, 1982; Voegele et al., 2001), the haustorium functions to secrete effector proteins into the host cell across the interface to suppress host defenses, including those elicited upon recognition of microbe-associated molecular patterns by cell surface receptors (Catanzariti et al., 2007). The formation and sustained functioning of the haustorium enables successful colonization of the plant by the invading pathogen, leading to establishment of a long-term parasitic relationship with the host.

Facing selective pressure imposed by the pathogens, plants have evolved postinvasin resistance mechanisms, often controlled by dominant resistance (*R*) genes, whose products directly or indirectly detect specific pathogen effectors and trigger effective defense responses (Chisholm et al., 2006; Jones and Dangl, 2006). *R* protein–triggered resistance to various pathogens is normally race specific and only effective against pathogen strains expressing the cognate effector protein recognized by the *R* protein. This resistance is often associated with a hypersensitive response (HR), which is manifested as rapid death of the invaded cell and in some cases a few surrounding cells (Hammond-Kosack and Jones, 1997; Morel and Dangl, 1997; Schulze-Lefert and Panstruga, 2003). Most characterized plant *R* proteins belong to members of the nucleotide binding site–leucine-rich repeat (NB-LRR) protein superfamily (Jones and Dangl, 2006). Several isolated powdery mildew resistance genes belong to this class. Those include the *R* genes at the *Mla*...
locus of barley (*Hordeum vulgare*; Halterman et al., 2001; Zhou et al., 2001; Halterman and Wise, 2004) and *Pm3b* of wheat (*Triticum aestivum*; Yahiaoui et al., 2004).

The two homologous *Arabidopsis thaliana* *R* genes, RPW8.1 and RPW8.2, from the Ms-0 accession confer broad-spectrum resistance to host-adapted *Golovinomyces* spp fungi belonging to the *Ascomycetes* (Xiao et al., 2001), which are the causal agents of powdery mildew disease on numerous dicot plant species (Braun, 1987). RPW8.1 and RPW8.2 are predicted to encode proteins that contain an N-terminal transmembrane and one to two coiled-coil domains (Xiao et al., 2001) and thus are placed in an unusual class of *R* genes (Dangl and Jones, 2001). Despite their atypical predicted protein structure, RPW8.1 and RPW8.2 (both together are referred to as RPW8 unless otherwise indicated) activate HR and other defense responses via the conserved salicylic acid (SA)- and ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1)-dependent signaling pathway also recruited by a subset of NB-LRR *R* proteins that contain an N-terminal TIR (for toll and interleukin-1 receptor) domain for race-specific resistance (Xiao et al., 2003, 2005). This implies that the broad-spectrum mildew resistance is specified by the RPW8 proteins upstream of SA-EDS1–dependent signaling.

In our efforts to address how RPW8 achieves broad-spectrum resistance, we discovered that expression of the RPW8.2 protein is highly induced by powdery mildew. Intriguingly, we also found that RPW8.2 is specifically targeted to the EHM during haustorium differentiation, whereby it may be involved in enhancing the formation of a callosic encasement of the haustorial complex (EHC) and in onsite accumulation of *H₂O₂*, possibly for conformation of a callosic encasement of the haustorial complex, whereby it may be involved in enhancing the distribution of the fluorescent signals was observed in Columbia-0 (Col-0) cells expressing the YFP protein alone under control of the 35S promoter (Figure 1D), indicating that the haustorium-associated localization of RPW8.2-YFP was determined by RPW8.2. Furthermore, compared with healthy, plump haustoria in cells of Col-0 expressing 35S::YFP (Figure 1D), RPW8.2-YFP–encased haustoria, often although not always, appeared deformed or shrunk and in some cases lacked PI staining (arrows in Figure 1E). These findings, together with the observation that RPW8 expression does not seem to affect fungal spore germination (see Supplemental Figure 2 online), suggest that RPW8.2 is specifically targeted to the periphery of the haustorium of *Gc UCS1* (i.e., the host-pathogen interface) to activate postinvasion haustorium-targeted resistance.

To see if the haustorium-specific targeting of RPW8.2 also occurs in interactions with nonhost powdery mildew pathogens, we introduced NP:RPW8.2-YFP into the *eds1-2* mutant, which exhibits compromised nonhost and host resistance (Aarts et al., 1998; Falk et al., 1999), and inoculated the plants with barley powdery mildew (*Blumeria graminis* f. sp hordei *[Bgh]*) for which *Arabidopsis* is a nonhost. Haustorium formation was observed for *Bgh*, albeit at a very low frequency. In cells invaded by a haustorium, RPW8.2-YFP was either found to be colocalized with a small haustorium or an outlayer half-encasing a bigger haustorium starting from the haustorial neck (Figures 2A and 2B), which is reminiscent of the observation that the callosic encasement of the HC (referred to as the EHC in this study) induced in *Arabidopsis–Bgh* interaction was incomplete and showed a similar inverted cup shape (Meyer et al., 2008).

Algae-like oomycete pathogens penetrate anticlinal cell walls in between neighboring epidermal cells (Slusarenko and Schlaich, 2003; Grenville-Briggs et al., 2008) and subsequently develop similar haustorial structures in mesophyll cells and occasionally epidermal cells, presumably also for nutrient uptake. We thus wondered if RPW8.2 is also targeted to the

**RESULTS**

**RPW8.2 Is Induced by Fungal Invasion and Targeted to the Host-Pathogen Interface**

In previous studies, the RPW8.1-yellow fluorescent protein (YFP) fusion expressed from the native promoter (NP) was mainly found in mesophyll cells and rarely if ever observed in epidermal cells, whereas RPW8.2-YFP was detected in both epidermal and mesophyll cells (Wang et al., 2007). Because powdery mildew pathogens invade only plant epidermal cells via direct penetration, in this study, we focused on the mode of action of RPW8.2 in the epidermal cells only. A small proportion (~5%) of RPW8.2-YFP transgenic lines developed spontaneous cell death associated with expression of RPW8.2-YFP in punctate spots, suggesting that similar to the wild-type protein (Xiao et al., 2003), RPW8.2-YFP retains the ability to trigger cell death (see Supplemental Figure 1A online). We selected transgenic lines (single transgene, T4 generation) showing no constitutive RPW8.2-YFP expression (Figure 1A) to examine the expression and localization of RPW8.2-YFP in leaves after inoculation of *Golovinomyces cichoracearum* UCSC1. Remarkably, we observed many sac-like fluorescent spheres in the size of 10 to 20 μm in the epidermal cells of the inoculated leaves (Figure 1B). Such fluorescent spheres were detectable at 18 to 20 h after inoculation (HAI), overlapping with the haustorial differentiation from 12 to 18 HAI (Koh et al., 2005). Visualizing the fungal structures by propidium iodide (PI) staining revealed that the fluorescent spheres were in the vicinity of fungal mycelia (see Supplemental Figure 1B online). Close examination revealed that the fluorescent signal from RPW8.2-YFP precisely colocalized with fungal haustoria (Figure 1C). More significantly, in cells accommodating a developing haustorium, small RPW8.2-YFP–positive vesicles of varied size could be seen to be moving toward and fusing into a membrane-like layer peripheral to the haustorium (arrowheads in Figure 1C; see Supplemental Movie 1 online). Initially, the RPW8.2-YFP–labeled fluorescent layer encasing an incipient haustorium appeared punctate and uneven; as the haustorium matured, the punctate layer progressively turned into a more evenly distributed coat encasing the haustorium (see Supplemental Figure 1C online). The global distribution of the fluorescent signals was observed in Columbia-0 (Col-0) cells expressing the YFP protein alone under control of the 35S promoter (Figure 1D), indicating that the haustorium-associated localization of RPW8.2-YFP was determined by RPW8.2. Furthermore, compared with healthy, plump haustoria in cells of Col-0 expressing 35S::YFP (Figure 1D), RPW8.2-YFP–encased haustoria, often although not always, appeared deformed or shrunk and in some cases lacked PI staining (arrows in Figure 1E). These findings, together with the observation that RPW8 expression does not seem to affect fungal spore germination (see Supplemental Figure 2 online), suggest that RPW8.2 is specifically targeted to the periphery of the haustorium of *Gc UCS1* (i.e., the host-pathogen interface) to activate postinvasion haustorium-targeted resistance.

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haustorium of oomycete pathogens. We examined 4-week-old, NP:RPW8.2-YFP–expressing seedlings inoculated with *Hyaloperonospora arabidopsidis* Noco2 at 2 d after inoculation (DAI) and observed accumulation of RPW8.2-YFP in the periphery of the oomycete haustoria (Figures 2C and 2D).

To exclude the possibility that localization of RPW8.2 to the host-pathogen interface is induced by membrane damage and the subsequent repair process during haustorial invasion, we wounded plants expressing NP:RPW8.2-YFP and found no induction or translocation of RPW8.2-YFP to the damaged PM, despite that RPW8.2-YFP was found in the host-pathogen interface within the same cell (see Supplemental Figure 3 online).

These observations suggest that it is the differentiation of the haustorium, rather than mechanical perturbation of the PM, that induces RPW8.2 translocation to the host-pathogen interface.

**RPW8.2 Is Localized to the EHM**

To determine the precise localization of RPW8.2 at the host-pathogen interface, we isolated individual haustorial complexes

**Figure 1.** RPW8.2 Is Targeted to the Periphery of the Fungal Haustorium.

Except as noted, Col-0 lines expressing *RPW8.2-YFP* from the native *RPW8.2* promoter (NP) were inoculated with *G. cichoracearum* UCSC1, directly examined under a Zeiss epifluorescence microscope ([A] and [B]), or stained with PI to visualize the fungal structures and imaged by CLSM at 20 to 42 HAI. Bars = 50 μm in (A) and (B) and 5 μm in (C) to (E).

(A) Lack of RPW8.2-YFP expression in inoculated leaf epidermal cells from 0 to 15 HAI.

(B) Detection of RPW8.2-YFP expression as sac-like fluorescent objects in inoculated leaf epidermal cells at 20 HAI.

(C) Precise localization of RPW8.2-YFP to the surface of a haustorium (H). Note that the YFP signal stops at the haustorial neck. Arrowheads indicate RPW8.2-YFP–containing vesicles.

(D) A haustorium in a host cell of Col-0 expressing 3SS:YFP. Note that the localization pattern of YFP is distinct from (C).

(E) Haustoria encased by RPW8.2-YFP became shrunken (white arrow) or aborted (lack of PI staining; yellow arrows).
(HCs), stained them with PI, and examined by confocal laser scanning microscopy (CLSM). The EHM, and the haustorial PM and cell wall, together with the extrahaustorial matrix in between, comprise the host-pathogen interface (Mackie et al., 1991; Szabo and Bushnell, 2001) (see Supplemental Figure 4 online). The interfacial EHM (indicated by an arrowhead in Figure 3A) is recognizable from an isolated HC from Col-0. Yellow fluorescence from RPW8.2-YFP was indeed precisely colocalized with the PI-positive EHM of a slightly shrunken HC isolated from Col-0 cells expressing RPW8.2-YFP (Figure 3B). Interestingly, a 5- to 10-μm-thick, weakly PI-positive layer was visible in some HCs isolated from RPW8.2-YFP cells (Figure 3C). This layer corresponds to the callosic EHC. When a portion of the RPW8.2-YFP-labeled layer of a single HC was photobleached, the fluorescence was largely recovered in 5 min (Figure 3D), similar to that seen in the PM labeled by green fluorescent protein (GFP):SIMIP (Cutler et al., 2000) (Figure 3E), indicating that this layer possesses membrane-specific fluidity. Next, we performed immunolocalization assays on leaf sections of inoculated NP:RPW8.2-YFP plants using an anti-GFP antibody conjugated to gold particles. Under transmission electron microscopy (TEM), the EHM is discernable as an electron-dense layer surrounding the HC (Gil and Gay, 1977). The observed shrinkage and deterioration of the haustoria targeted by RPW8.2-YFP (Figure 1E) may account for the poor preservation of the haustorial structure observed by TEM (Figures 4A and 4B). Nevertheless, immunogold-labeled particles were found mainly at or attaching to the discernable EHM in sections of leaf samples from plants expressing RPW8.2-YFP (Figures 4A and 4B) but not in the negative control of Col-0 (Figures 4C and 4D), indicating that the immunogold labeling was specific and RPW8.2-YFP was indeed localized at the EHM. Some clustered gold particles were also detected in the cytoplasmic side of the deformed HC where the EHC resides (Figure 4B), likely representing RPW8.2-YFP vesicles. Based on these observations, we conclude that RPW8.2 is induced and specifically targeted to the EHM during haustorial differentiation of Gc UCSC1.

**RPW8.2 EHM Localization Is Independent of SA Signaling but Requires Functions of the Actin Cytoskeleton**

RPW8-mediated resistance engages SA-signaling (Xiao et al., 2003). We thus asked if SA signaling is necessary for targeting RPW8.2 to the EHM. For this, we introduced NP:RPW8.2-YFP in Col-0 expressing NahG (a bacterial gene encoding an SA hydrolase that depletes SA) or containing mutations eds1-2, eds5-1, pad4-1, or npr1-1 (each of which compromises SA signaling) by stable transformation. We detected RPW8.2-YFP expression and translocation to the EHM (see Supplemental Figure 5A online) in 15 to 25% of the transgenic lines, but most (>95%) of these plants were fully susceptible to Gc UCSC1. Thus, despite its critical role in RPW8-mediated defense, SA signaling is not required for targeting RPW8.2 to the EHM.

The actin cytoskeleton is required for vesicle transport (Lanzetti, 2007), reorganized in host cells challenged by powdery mildew or oomycete pathogens (Takekoto et al., 2003; Opalski et al., 2005), and required for resistance against powdery mildew and oomycete pathogens (Yun et al., 2003; Miklis et al., 2007). We thus investigated whether specific targeting of RPW8.2 to the EHM requires normal function of the cytoskeleton. We infiltrated cytochalasin E, an inhibitor of actin polymerization, or oryzalin, which...
Figure 3. RPW8.2 Is Targeted to the EHM.

Leaves of Col-0 or Col-0 transgenic for NP-RPW8.2-YFP were inoculated with G. cichoracearum UCSC1 and used for haustorium isolation at 2 DAI. Isolated haustoria were stained with PI and imaged by CLSM. Bars = 5 μm.

(A) A single optical section of an isolated HC from Col-0. Note the weakly PI-stained EHM (arrowhead) and numerous lobes emanating from the main body of the haustorium.

(B) A single optical section of an isolated HC showing precise colocalization of RPW8.2-YFP with the PI-stained EHM.

(C) An isolated HC showing localization of RPW8.2-YFP to the outer surface of the HC. Note the lightly PI-positive encasement of the HC (EHC).

(D) and (E) Fluorescence recovery after photobleaching on RPW8.2-YFP–labeled EHM in planta (D) and the GFP-SIMIP-labeled PM (E). Arrowheads point to the site of photobleaching.
an inhibitor of microtubule polymerization, into Gc USCS1–inoculated leaves of a Col-0 transgenic line homozygous for one copy of NP:RPW8.2-YFP (designated R2Y4) at 12 HAI and counted the number of RPW8.2-YFP–labeled haustoria under an epifluorescent microscope from 32 to 36 HAI and subsequently stained the leaves by trypan blue for visualization and counting of all haustoria. We found that compared with buffer control, cytochalasin E treatment significantly reduced EHM localization of RPW8.2-YFP in a dose-dependent manner, with the ratios of RPW8.2-YFP–labeled haustoria versus the total haustoria ranging from 40% (2 μg/mL cytochalasin E) to only 15% (8 μg/mL cytochalasin E) compared with 70% for buffer control (Figure 5A). The reduction of RPW8.2-YFP’s EHM localization was often accompanied with irregular localization of RPW8.2-YFP peripheral to the PM of the haustorium-invaded epidermal cells and in punctate spots in mesophyll cells underneath and fungus-induced cell death (see Supplemental Figure 6A online). By contrast, 31% (22 of 71 T1 lines and their T2 progenies) of the 35S:ADF6 transgenic lines exhibited reduced plant stature and developed spontaneous cell death in leaves (see Supplemental Figure 6B online). The development of cell death was preceded by expression of RPW8.2-YFP in punctate spots and H2O2 accumulation (Figures 5B and 5C). More significantly, localization of RPW8.2-YFP to the EHM was greatly reduced in these 35S:ADF6 lines with cell death (Figure 5D). RT-PCR showed that ADF6 was indeed expressed at higher levels in the lines that exhibited cell death, reduced stature, and compromised EHM localization of RPW8.2-YFP (see Supplemental Figure 6C online). However, we did not observe any detectable inhibition of RPW8.2-YFP’s localization to the EHM in the 35S:ADF5 lines. This result, together with a previous report that while ADF6 is expressed ubiquitously, ADF5 expression is restricted to root tip meristem (Dong et al., 2001), implies a likely functional diversification between ADF5 and ADF6. Combined, these results demonstrated that specific targeting of RPW8.2 to the EHM is largely actin cytoskeleton dependent and microtubule independent.

**Figure 4.** RPW8.2-YFP Localization to the EHM Is Revealed by Immunogold Labeling Using Anti-GFP (Which Also Detects YFP) and TEM.

(A) A lower-magnification image of a haustorium (H) in a cell of a transgenic Col-0 line expressing RPW8.2-YFP. Note the encasement of the haustorial complex (EHC). Bar = 1 μm.

(B) A close-up of the squared section in (A) showing that the immunogold particles (10 nm) were specifically localized at the EHM (arrowheads) near a haustorial lobe (L). Note a few particles (gray arrows) likely representing RPW8.2-YFP vesicles in the EHC region. Bar = 0.2 μm.

(C) and (D) Sections of a haustorium in a wild-type Col-0 cell at the same magnification as in (A) and (B), respectively.
The PEN1 syntaxin contributes to preinvasion (penetration) resistance of plants against nonadapted powdery mildew pathogens and was recently found to be enriched in the EHC along with several other associated SNARE proteins (Collins et al., 2003; Kwon et al., 2008; Meyer et al., 2008). We thus tested if the PEN1-dependent exocytosis pathway contributes to docking and fusion of RPW8.2 vesicles with the EHM. We first introduced NP:RPW8.2-YFP into the pen1-1 background and found that RPW8.2-YFP’s translocation to the EHM was grossly unaffected (see Supplemental Figure 5A online). We then generated plants expressing RPW8.2-DsRed and GFP-PEN1 in the pen1-1 background and found that in addition to the PM and the papillae as reported (Assaad et al., 2004; Bhat et al., 2005), GFP-PEN1 was indeed found in the EHC but had no obvious overlapping with the RPW8.2-DsRed–labeled EHM (see Supplemental Figure 5B online). Neither did we observe colocalization of GFP-PEN1 with RPW8.2-YFP in punctate spots. These observations suggested that a PEN1-independent/redundant, targeted protein-trafficking pathway is recruited for docking and fusion of RPW8.2 vesicles with the EHM.

**RPW8.2 Enhances the Formation of the Callosic Encasement of the HC**

The EHM-specific localization of RPW8.2 provides a perfect physical explanation for its broad-spectrum mildew resistance against *Golovinomyces* spp (Xiao et al., 2001). This further prompted us to ask how RPW8.2’s EHM localization contributes to antifungal defenses at the host-pathogen interface. To address this question, we examined trypan blue–stained, haustorium-invaded epidermal cells of Col-0, Ms-0, and Col-0 lines transgenic for RPW8 (line S5; see Methods) or NP:RPW8.2-YFP (line R2Y4) or 35S:YFP (as control) at 42 and 120 HAI. We detected three types of haustorium-invaded cells: (1) viable cells containing a deformed or shrunken haustorium with a 5- to 10-μm-thick, callose-rich layer designated EHC, which was discernable after trypan blue/aniline blue staining (indicated by black arrows in Figure 6A; top panel of Figure 6B); (2) cells undergoing HR (indicated by the red arrow in Figure 6A, note that the EHC may have developed in these cells); (3) viable cells with a healthy haustorium without the EHC (indicated by green arrows in Figure 6A; bottom panel of Figure 6B, note the lack of an EHC but presence of a papilla). We found that Ms-0, S5, and R2Y4, which were resistant to powdery mildew had 8 to 10 times higher ratios of type (1) + type (2) cells than Col-0 or Col-0 expressing YFP alone in both assays conducted at 42 or 120 HAI (Figure 6C).
Figure 6. RPW8 Enhances the Formation of the EHC and Accumulation of H$_2$O$_2$ in the HC.

Plants of indicated genotypes, including Col-0 lines transgenic for NP:RPW8.2-YFP (R2Y4) or RPW8.1 and RPW8.2 (S5), were inoculated with G. cichoracearum UCSC1 and subject to trypan blue (TB)/aniline blue (AB)/3,3'-diaminobenzidine (DAB) staining to visualize dying/dead host cells and fungal structures (TB), callose deposition (AB), and H$_2$O$_2$ accumulation (DAB), respectively.

(A) Representative leaf sections showing three types of invaded cells: cells containing a haustorium with (black arrows) or without the EHC (green arrows) and cells undergoing HR (red arrow) at 5 DAI. Mycelia were removed to aid visualization of haustoria. Bars = 50 μm.

(B) Single representative cells containing a shrunken haustorium with the EHC (top panel) or a healthy haustorium without EHC (bottom panel). Note the callose (stained blue by AB) deposition in the EHC and at the penetration site (i.e., the papilla; arrow). Bars = 20 μm.

(C) and (D) Types and frequencies of host-pathogen interactions at 42 and 120 HAI. White, light-gray, and dark-gray bars indicate frequencies of living cells containing a haustorium without an EHC, living cells containing a haustorium with an EHC, and cells undergoing HR, respectively. Data are means ± se, calculated from three duplicated experiments. Asterisks indicate values significantly different from that of Col-0 (C) or S5 (D) (P < 0.01; n = 3, Student’s t test).

(E) Perturbation of the integrity of the EHC by pmr4-1 results in more frequent activation of cell death in the RPW8 background at 48 HAI (top panel; also see [D]), which is preceded by H$_2$O$_2$ release from the HC into the cytoplasm (reflected by reddish brown precipitates; bottom panel) at 36 HAI. Bars = 10 μm.
RPW8.2 Enhances Accumulation of H2O2 in the HC

It has been established that H2O2 production and accumulation precedes HR cell death and is associated with RPW8 resistance (Xiao et al., 2001, 2003). To investigate how H2O2 is spatiotemporally involved in resistance in relation to RPW8.2’s EHM localization and subsequent EHC formation, we measured H2O2 accumulation in single invaded epidermal cells. H2O2 accumulation in the invaded epidermal cells of Ms-0, S5, or R2Y4 was first detected around 27 HAI at a similar time as or slightly later than the first appearance of the EHC. Three general patterns concerning H2O2 accumulation were observed in the time frame from 27 to 72 HAI: (1) in the HC, (2) in the whole invaded cell, and (3) no apparent H2O2 accumulation (see Supplemental Figure 7C online). Although there may not be a strict correlation between EHC formation and H2O2 accumulation, localized H2O2 accumulation in the HC was always accompanied by EHC formation. This suggests that the EHC is necessary (though may not be sufficient) for localized H2O2 accumulation in the HC. To test this possibility, we introduced a loss-of-function mutation (pmr4-1) of PMR4/GSL5 encoding a glucan synthase-like membrane protein known to be required for onsite callose accumulation in response to haustorial invasion (Jacobs et al., 2003; Nishimura et al., 2003) into the transgenic lines S5 (top panel of Figure 6E) to perturb the structural integrity of the EHC. As shown by representative images in the bottom panel of Figure 6E, in haustorium-invaded pmr4-1/S5 cells, H2O2 was often found to be in the cytoplasm even though it was more enriched in the HC. By contrast, H2O2 in the infected pmr4-1 cells showed less but more even distribution, while H2O2 in S5 cells was often HC confined. The increased frequency of whole-cell H2O2 accumulation correlated with a much higher percentage (>90%) of cells undergoing HR in pmr4-1/S5 compared with pmr4-1 or S5 alone (Figure 6D). These results suggest that RPW8.2 may trigger production and/or accumulation of H2O2 and concomitantly enhance EHC formation to confine H2O2 (perhaps other toxic defense compounds) in the HC, thereby constraining the haustorium of powdery mildew while reducing oxidative damage to the host cell itself.

RPW8.2 Proteins Encoded by Susceptible Alleles Are Either Incapable of Activating Defense or Less Efficient in Trafficking to the EHM

The majority of Arabidopsis accessions are susceptible or moderately susceptible to powdery mildew Gc UCSC1 (Adam et al., 1999; Orgil et al., 2007). To test if there is a correlation between functional diversification/deterioration of the RPW8.2 alleles and the mildew-susceptible phenotypes, we transgenically analyzed the localization and function of RPW8.2 encoded by four alleles (Db-0, Fm-3, Bu-23, and Gb-1), each representing a distinct subgroup of divergent RPW8.2 alleles identified in Arabidopsis accessions with a susceptible or intermediate disease phenotype (see Supplemental Figure 8 online; note that Gb-1 had been scored as resistant [Orgil et al., 2007] but reclassified as intermediate in this study after repeated careful infection tests). Col-0 transgenic lines expressing RPW8.2x-YFP (X denotes any of the four alleles) from the same native RPW8.2Ms-0 promoter were examined (>120 T1 individuals and 20 independent T2 progenies for each construct) for disease reaction phenotypes and protein localization. All transgenic plants expressing NP:RPW8.2Ds-0/Fm-3/Bu-23-YFP showed strong spontaneous cell death before infection and were susceptible to Gc UCSC1; however, EHM localization of the corresponding fusion protein was readily detectable in 30 to 49% of those lines (Figure 7A). By contrast, 41% of Col-0 lines expressing NP:RPW8.2Db-1-YFP exhibited strong spontaneous cell death in rosette leaves and reduced plant stature (Figure 7B), which were associated with constitutive expression of RPW8.2Db-1-YFP in punctate spots (data not shown), and 22% of the lines did not show spontaneous cell death but developed Gc UCSC1–induced massive cell death at 5 to 7 DAI, 2 to 3 d slower than the development of normal HR (Figure 7C). Examination of the infected leaves of those lines showed that RPW8.2Db-1-YFP was often detected in punctate spots in the invaded epidermal cells and rarely found in the EHM (Figure 7A). Similar to plants of the Gb-1 accession (see Supplemental Figure 8 online), Col-0 plants transgenic for NP:RPW8.2Db-1-YFP supported moderate fungal growth despite development of massive fungus-induced cell death at 8 DAI (Figure 7C). These observations suggested that RPW8.2Db-1 (and those encoded by RPW8.2Ds-0/YFP-like alleles) may be more active than RPW8.2Ms-0 in triggering cell death and/or less efficient in EHM localization and, therefore, less effective in restricting fungal growth.

DISCUSSION

In this study, we have revealed that RPW8-mediated broad-spectrum mildew resistance involves specific targeting of...
RPW8.2 to the host-fungal interfacial membrane and onsite activation of defenses against the invading pathogen. This finding has three important implications. First, the mysterious EHM mostly likely is of host origin and de novo synthesized. Second, plants have evolved elaborate mechanisms to activate defense at the host-pathogen interface to achieve broad-spectrum resistance against haustorium-forming pathogens. Third, there may exist an EHM-targeted protein trafficking pathway in plants for defense against haustorial invasion.

**RPW8.2, an EHM-Specific Protein**

The haustorium is a highly specialized feeding structure of plant pathogens falling into taxonomically distinct and agriculturally important species, including the fungal powdery mildew (Ascomycetes) and rusts (Basidiomycetes), and the fungus-like oomycetes. Concrete evidence has been provided for the two conceptual functions of the haustorium in pathogenesis: nutrient uptake from the host (Hahn and Mendgen, 2001; Szabo and Bushnell, 2001) and secretion of effectors into the host cell to suppress host defense (Catanzariti et al., 2007). However, the molecular warfare at the host-pathogen interface remains largely uncharacterized.

Previous searches for EHM-resident proteins in the field had limited success. Using a monoclonal antibody raised against the isolated HCs in pea (*Pisum sativum*), a glycoprotein (250 kD) was detected to be specifically localized to the periphery of the HC where the EHM was thought to form (Roberts et al., 1993). However, the identity and origin of this glycoprotein still remains to be resolved. More recently, Koh et al. (2005) examined eight *Arabidopsis* PM resident proteins in powdery infected leaf epidermal cells and found that they were all absent from the EHM. These observations suggest that the EHM by nature may be distinct from the host cell PM.

In this study, we revealed that RPW8.2 is specifically targeted, most likely via intracellular vesicle transport, to the EHM during haustorium biogenesis. The highly specific localization of RPW8.2 to the EHM but not to the host PM also lends support to earlier observations that the EHM and the haustorial PM are sealed at the neck of the HC by two regions, termed neckbands, that serve to separate the EHM and the extrahaustorial matrix from the host PM and the apoplastic space (Gil and Gay, 1977; Gil and Gay, 1979; Gil and Gay, 1980).

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**Figure 7.** Both Defense Activation and EHM Localization Are Required for RPW8.2 to Confer Resistance.

The RPW8.2 alleles from the indicated accessions were translationally fused with YFP at the C terminus, and all the constructs were expressed from the promoter of RPW8.2*Ms-0* in Col-0. T2 plants are shown. (A) Representative images showing typical localization of RPW8.2 (fused to YFP) encoded by the indicated alleles (top) at 2 DAI. The disease reaction (DR) scores (0, resistant; 1 to 2, intermediate [I]; 2 to 3 or 3, susceptible [S]; Xiao et al., 2005) are shown below the images. H, haustorium. Arrowheads indicate RPW8.2*Bg-1*-YFP in punctate spots. Bars = 10 μm. (B) Representative 5-week-old T2 plants before inoculation. Note the spontaneous cell death and reduced plant stature in plants of one representative RPW8.2*Bg-1*-YFP line. (C) Representative leaves of the transgenic lines expressing the indicated RPW8.2 allele in comparison with a leaf of Col-0 (c) inoculated with Gc UCSC1 at 8 DAI. Note the induced massive cell death and fungal growth in the same leaf expressing RPW8.2*Bg-1*-YFP.
Bushnell and Gay, 1978). To our knowledge, the highly interface-specific localization of RPW8.2 is most striking but similar to the translocation of Nramp1 (for natural resistance-associated macrophage protein 1) and Lamp1 (for lysosomal-associated membrane protein 1) in activated animal macrophages to the host-pathogen interfacial (phagosomal) membrane from the late endocytic compartments (late endosome/lysosome) of resting macrophages (Searle et al., 1998; Canonne-Hergaux et al., 1999; Huynh et al., 2007). Our finding demonstrates that the EHM is indeed a special interfacial membrane likely of host origin that may be de novo synthesized and/or modified by the host and the pathogen for defense and pathogenesis, respectively. Thus, RPW8.2 could be used as a tool to investigate the molecular differentiation and composition of the EHM and the targeted-defense mechanism of the host.

It has recently been reported that subsequent to the perception of the cognate effector molecules from the pathogens, a small fraction of the NB-LRR proteins MLA10, N, and RPS4 is translocated from the cytoplasm to the nucleus to activate immune responses (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007). NPR1, the central regulatory protein of systemic acquired resistance, undergoes SA-induced oligomer-to-monomer conformational change and is subsequently translocated from the cytoplasm to the nucleus whereby it activates transcription of pathogenesis-related genes (Mou et al., 2003; Tada et al., 2008). By contrast, RPW8.2 is directly deployed at the host-pathogen battleground to fight against the invading haustorium. How RPW8.2 is connected to SA signaling to activate defenses at the interface remains to be elucidated.

Two Functions of RPW8.2 Both Contribute to Resistance

Targeting of RPW8.2 to the EHM for onsite activation of defenses against the invading haustorium provides a logical mechanistic explanation for RPW8-mediated broad-spectrum mildew resistance. Our genetic data suggest that trafficking of RPW8.2 to the EHM per se does not seem to require SA signaling because impairing the SA pathway did not affect EHM localization of RPW8.2-YFP (see Supplemental Figure SA online). On the other hand, EHM localization is not a prerequisite for RPW8.2 to trigger SA-dependent H2O2 production and cell death in the absence of pathogens (Xiao et al., 2003; see Supplemental Figure 1A online). In fact, inhibition of RPW8.2-YFP trafficking to the EHM by impeding actin cytoskeleton function resulted in H2O2 accumulation and cell death (Figure 5; see Supplemental Figure 6 online). Thus, RPW8.2 appears to be equipped with two intrinsic functions, EHM-specific localization and activation of H2O2 production/accumulation, each of which is controlled by a different pathway. It is conceivable that these two functions must be properly coordinated to achieve haustorium-targeted resistance against powdery mildew pathogens with minimum host penalty. This is evidenced by the observations that RPW8.2 alleles from Arabidopsis accessions that are not resistant to Gc UCSC1 were found to encode proteins either unable to activate defense at the EHM (for the Dra-0, FM-3, and Bu-23 alleles) or less efficient in EHM localization (for the Bg-1 allele) (Figure 7). One may wonder why these divergent alleles have been maintained in the Arabidopsis populations. A plausible explanation is that expression of RPW8.2Ms-0 (due to activation of SA signaling upon insults from other pathogens) is costly in the absence of powdery mildew pathogens, hence, the generation and maintenance of RPW8.2 alleles defective in H2O2 production to reduce the cost associated with RPW8.2 expression (Orgil et al., 2007). However, we found it difficult to explain the generation and maintenance of alleles that encode RPW8.2 more active in triggering cell death and/or less efficient in trafficking to the EHM. One possibility is that such RPW8.2 alleles may serve another (defense) function that may benefit the plant under certain environmental conditions and hence have been preserved in the populations.

Dra-0 and Fm-3 differ from Ms-0 in RPW8.2 by only three to five amino acids (see Supplemental Figure 8A online). This points to a potential role of these residues (especially the T645, D116G and T161K sites) in the activation of SA-dependent defense. The Bu-23 allele lacks the C-terminal 31 amino acids. This suggests that at least the C-terminal 31 amino acids are dispensable for EHM targeting. RPW8.2Ms-0 differs from RPW8.2Ms-0 in 13 amino acids. Apart from the five present in RPW8.2Ms-0, most of the additional substitutions are located in the N-terminal 80–amino acid region. These substitutions may render the protein more active in cell death activation and/or less competent in trafficking. A detailed structure-function analysis is needed to map the domain/residue in RPW8.2 for the defense and trafficking functions.

Another intriguing feature of RPW8.2-mediated, haustorium-targeted defense is the concomitant EHC formation and H2O2 production/accumulation in the HC (Figure 6; see Supplemental Figure 7B online). The callosic EHC may serve several functions during defense. First, it may present a physical barrier to block the intrusion of the haustorium, the delivery of pathogen effectors into the host cell, and possibly the nutrient supply to the pathogen. Second, similar to papillae, the EHC may provide a structural scaffold to contain and enrich toxic molecules produced by the host cell to poison the haustorium, while protecting the host cell from the toxic molecules produced by the host (e.g., H2O2) or the pathogen at the interface. The speculation on the latter function is in line with our observation that perturbation of the EHC by pmr4-1 resulted in massive host cell death associated with fungus-induced H2O2 accumulation in the invaded epidermal cells (Figures 6D and 6E). It should be pointed out that the enhanced HR in pmr4-1 mutant plants may be caused by elevated SA signaling due to the pmr4-1 mutation (Nishimura et al., 2003) and the resultant amplification of RPW8-triggered defense response. However, there may exist a mechanistic connection between H2O2 accumulation in the cytoplasm of the invaded cells due to structural perturbation of the EHC (and papillae) and upregulation of SA signaling. Subcellular callosic structures similar to the EHC have long been observed in other host–pathogen interactions (Heath and Heath, 1971; Bushnell, 1972; Donofrio and Delaney, 2001; Mellersh and Heath, 2003; Soylu et al., 2003), suggesting that EHC formation may be a general basal defense mechanism against haustorial invasion and a target for suppression by the pathogens for pathogenesis. Based on the correlation between RPW8.2 expression and H2O2 accumulation in the HC along with EHC formation, one can envision an exquisite postinvasion defense mechanism in Arabidopsis that involves targeting RPW8.2 to the host-pathogen
interface to enhance H$_2$O$_2$ accumulation and EHC formation for
constraining the haustorium while protecting the host cell itself.
However, because there is rather frequent HR observed in
RPW8-expressing plants, especially in high light conditions,
the HR may serve as a next, perhaps more destructive, step
to reinforce the earlier defense exerted by H$_2$O$_2$ in the HC that
may not be sufficient to contain the adapted H$_2$O$_2$-tolerant
pathogens.

Existence of an EHM-Targeted Protein Trafficking Pathway?

Polarized protein trafficking plays essential roles in multiple
processes of plant development, including cell division, pollen,
and root hair tip growth (Bednarek and Falbel, 2002; Cole and
Fowler, 2006; Yang, 2008). Specific targeting of RPW8.2 to the
EHM suggests that there may exist a highly specific, EHM-
targeted protein trafficking pathway recruited for defense
against haustorial invasion and that there must be a targeting
signal in RPW8.2 that is recognized by the trafficking machinery
to guide the RPW8.2-residing vesicles to dock to and fuse with
the EHM.

The PEN1 syntaxin defines a protein secretion pathway that
plays an essential role in preinvasion resistance (Collins et al.,
2003). Its focal accumulation in the papillae agrees well with this
function (Assaad et al., 2004). Recently, PEN1 and its SNARE
partner SNAP33 were also found in the EHC (Meyer et al., 2008),
implying a role of these proteins for resistance against haustorial
invasion. To date, the cargo(s) of the PEN1-defined polarized
secretion pathway has not been identified. Our genetic analysis
suggested that PEN1 is either independent of, or redundant for,
targeting of RPW8.2 to the EHM (see Supplemental Figure 5
online). Based on the observations that PEN1 (and its interacting
partner SNAP33) were selectively incorporated into papillae and
the EHC (Assaad et al., 2004; Meyer et al., 2008) but not in the
EHM where RPW8.2 is localized, we speculate that (1) at least
two separate polarized/targeted protein trafficking pathways are
activated during haustorial invasion, (2) RPW8.2 is unlikely to be
a cargo of the PEN1-dependent exocytosis pathway, and (3) a
syntaxin(s) other than PEN1 may be recruited for the fusion of
RPW8.2-containing vesicles with the EHM. Thus, RPW8.2 can be
used as a reporter protein to investigate the yet-to-be charac-
terized, EHM-targeted protein trafficking pathway evolved in
plants for defense.

Another challenging question for future studies is: what is the
signal that activates the EHM-targeted protein trafficking path-
way? Because RPW8.2 is targeted not only to the EHM induced
by the well-adapted Gc UCSC1, but probably also to the EHM
induced by the nonadapted barley powdery mildew or the
oomycete pathogen Hp Noco2, we hypothesize that during the
haustorium differentiation the host cell may be able to detect
the presence of a microbe-associated molecular pattern from
these pathogens and/or sense the pathogen-triggered lipid/
protein modification of the invaginated PM (i.e., the EHM) and
subsequently activate an EHM-specific lipid/protein trafficking
pathway that is co-opted for targeting defense molecules, includ-
ing RPW8.2 to the EHM. If so, it is conceivable that well-adapted
haustorium-forming pathogens may actively suppress/interfere
this targeted trafficking pathway. Alternatively, the pathogens
may hijack the host trafficking machinery to deliver lipids and
potentially proteins to the EHM to allow infection, and subse-
quently RPW8.2 has evolved to use this process to focus the
defense response. The first scenario could provide an explana-
tion that not all haustoria of Gc UCSC1 were found to be labeled
by RPW8.2-YFP in plants expressing NP:RPW8.2-YFP at a given
time point (Figure 5A) and that some haustoria in RPW8-
expressing plants lacked the EHC and appeared normal (Figures
6A and 6C). Accordingly, because RPW8.2-YFP was only rarely
found to be targeted to the oomycete HC, it is possible that Hp
Noco2 may suppress RPW8.2’s trafficking to the EHM to a
greater extent than does Gc UCSC1. Alternatively, the expres-
sion level of RPW8.2-YFP may be too low to allow visualization
of RPW8.2-YFP in the EHM induced by Hp Noco2 because RPW8
transcription is known to be attenuated by high relative humidity
(>90%) conditions required for Hp Noco2 infection (Xiao et al.,
2003; Wang et al., 2007). It remains to be determined if RPW8.2
can be targeted to the interfacial membrane induced by other
haustorium-forming pathogens, such as rust fungi or nonhaus-
torium fungal pathogens. If so, RPW8.2 may be engineered as a
delivery vehicle to target a wide range of antimicrobial peptides
to the host-pathogen interface to induce novel and more effec-
tive resistance.

METHODS

Plant Lines, Growth Conditions, and Transformation

Arabidopsis thaliana accession Col-0 was used for generation of trans-
genic lines expressing the RPW8.2-YFP fusion construct from the native
RPW8.2 promoter (NP). Accession Ms-0 containing RPW8.1 and RPW8.2
(together referred to as RPW8 unless otherwise indicated) and/or a homozygous
Col-0 transgenic line S5 carrying a single copy of RPW8 under control of the native promoters (Xiao et al., 2003) were used as
resistance references in the pathogen tests. The following transgenic or mutant lines were used for NP:RPW8.2-YFP transgenic studies and/or
genetic crossings with S5: eds1-2, pad4-1, NahG, pen1-1, and pmr4-1. Except eds1-2, which is from accession Landsberg erecta, all other lines
have the Col-0 genetic background.

Unless otherwise indicated, seeds were sown in Sunshine Mix #1
(Maryland Plant and Suppliers) and cold treated (4°C for 2 d), and
seedlings were kept under 22°C, 75% relative humidity, short-day (8 h
light at ~125 µmol·m$^{-2}$·s$^{-1}$, 16 h dark) conditions for 5 to 6 weeks before
pathogen inoculation or other treatments.

DNA Constructs and Transcription Analysis

Plasmids for NP:RPW8.1-YFP and NP:RPW8.2-YFP were made in a
previous report (Wang et al., 2007). Alleles of RPW8.2 from Dra-0, Fm-3,
Bu-23, and Bg-1 (Orgil et al., 2007) were amplified with the same primers
for the Ms-0 allele and C-terminally in-frame fused with YFP. The chimeric
genes were placed downstream of the RPW8.2 promoter in the
pZPR823 binary vector (Wang et al., 2007). For overexpression of ADF5
(Atf316700) and ADF6 (Atf31200), the genomic sequence of the two
genes and their 3’ untranslated regions were PCR amplified and cloned
into pEarleyGate 100 under control of the 35S promoter (Earley et al.,
2006). All the DNA constructs were introduced into Arabidopsis Col-0,
R2Y4 (Col-0 line transgenic for NP:RPW8.2-YFP), or various mutant lines
via Agrobacterium tumefaciens-mediated transformation using the A.
tumefaciens strain GV3101 (Clough and Bent, 1998). For measuring
transcript levels, total RNA was isolated from leaves or seedlings with TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from 2 μg of total RNA with reverse transcriptase using random hexamers. Relative mRNA levels were determined by PCR using equal amounts of total cDNA, and the PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining. After the amplification was optimized to be in the logarithmic range, ADF5, ADF6, and UBC21 (At5g25760; the endogenous control) were amplified for 30, 30, and 28 cycles, respectively, using gene-specific primers (see Supplemental Table 1 online).

Pathogen Infection and Microscopy Analyses

The powdery mildew isolate Golovinomyces cichoracearum UCSC1 was maintained on live eds1-2 or pad4-1 plants for generation of fresh inoculums. Inoculation and visual scoring of disease reaction phenotypes were done as previously described (Xiao et al., 2005). Fungal structures and dead cells in inoculated leaves were visualized with trypan blue staining (Xiao et al., 2003) at 42 HAI or 5 DAI. Interaction sites were counted and classified into three categories: (1) viable cells with a deformed or shrunken haustorium encased by a callose-containing layer known as the EHC (Jacobs et al., 2003); (2) cells undergoing haustorium-induced HR; and (3) viable cells with a healthy haustorium without the EHC. At least 300 interaction sites per genotype were scored at 42 HAI and 5 DAI in each of the three duplicate experiments. Student’s t test was performed to test statistical significance. In situ detection of callose was performed to test statistical significance. In situ detection of callose was done by aniline blue (0.01% in an aqueous solution containing 150 mM KH₂PO₄, pH 9.5) staining. In situ detection of H₂O₂ was performed as previously described (Xiao et al., 2003). Single or multiple channel epifluorescent images were acquired with a Zeiss Axio microscope coupled with an HBO 100 microscope illumination system.

Intact HCs were isolated from Gc UCSC1-infected Col-0 and NP-RPW8.2-YFP transgenic plants at 2 DAI according to a published protocol (Gil and Gay, 1977) with slight modification. Briefly, ~2 g of infected Arabidopsis leaves were collected at 5 DAI and gently homogenized using a pestle in a mortar in 10 mL of isolation buffer (0.1 M phosphate buffer, pH 6.7, 0.1 M sucrose, and 1.5 mM MgSO₄). The suspension was filtered through a 40-μm nylon mesh and centrifuged at 3000 g for 10 min at 4°C. The pellet was resuspended in 5 mL of isolation buffer and filtered through a 15-μm nylon mesh. The filtrate was centrifuged at 2000 g for 10 min at 4°C. Then, the pellet was resuspended in 4 mL of isolation buffer, layered over 10 mL of glycerol (70%, v/v) in phosphate buffer (0.1 M, pH 6.7), and centrifuged at 800 g for 10 min at 4°C. The upper dark-green layer was discarded, and the central zone (directly below the upper layer) containing the HCs was removed, diluted with 2.5 mL of the phosphate buffer, and centrifuged at 4750g for 10 min 4°C. The sediments (HCS) were washed with the phosphate buffer three times, followed by centrifugation, and finally resuspended in 0.3 mL of phosphate buffer. Freshly isolated HCs were diluted in isolation buffer, stained with 0.5% PI solution, and visualized by CLSM. CLSM images were acquired as previously described (Wang et al., 2007). All pictures presented in the figures are projections from Z-stacks of 15 to 40 images unless otherwise indicated. The image data were processed using Zeiss LSM Image Browser or LSM5 Image Examiner and Adobe Photoshop.

Electron Microscopy and Immunogold Labeling

Plants of Arabidopsis Col-0 and Col-0 transgenic for NP-RPW8.2-YFP (line R2Y4) were heavily inoculated with Gc UCSC1. Leaves were sampled at 5 DAI and cut into 2 × 3-mm sections, fixed in fixative containing 3% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, overnight at 4°C. The fixed tissues were rinsed three times in 0.1 M phosphate buffer for 15 min. After dehydration in a gradient of ethanol, the tissues were sequentially infiltrated with 2:1, 1:1, and 1:2 mixture of ethanol:LR White overnight at 4°C. Then, the samples were transferred in three changes of pure LR White for 1 h each at room temperature and kept in the last change of LR White overnight or stored at 4°C.

The samples were embedded in a flat embedding mold and cured in 60°C oven for 24 h. Ultrathin sections were put on Formvar carbon-coated 200 mesh nickel grids. The grids were incubated in 1% H₂O₂ for 10 min to remove the antigen and washed twice in distilled water. Then, the grids were incubated for 15 min in 0.05 M glycine prepared with 0.1 M PBST buffer (10 mM phosphate buffer, 150 mM NaCl, pH 7.4, 1% BAS, and 0.15% Tween 20) to inactivate the residual aldehyde groups present after aldehyde fixation. Nonspecific binding sites were blocked for 30 min with 5% goat serum in PBST buffer. Immunogold labeling was performed using rabbit anti-GFP serum, which can also detect YFP (1:200, ab290; Abcam) overnight at 4°C and goat anti-rabbit coupled to 10-nm gold particles (1:50; EMS) at 4°C overnight. After contrast staining with 2.5% uranyl acetate for 5 min, samples were examined and images were acquired with a Zeiss EM10 transmission electron microscope. Plate films were used to record the TEM images, which were then scanned to convert into digital images.

Pharmacological Treatments

Fully expanded leaves of 7-week-old RPW8.2-YFP plants were detached from the base of the petioles and inserted into Murashige and Skoog agar medium in Petri dishes. The detached leaves were then evenly inoculated with Gc UCSC1 using a settling tower. At ~12 HAI, each leaf was pressure-infiltrated with a syringe lacking a needle and solution containing 0, 1, 2, 4, or 8 μg/mL of cytocalasin E (Sigma-Aldrich) or 0, 60, 120, or 180 μg/mL of oryzalin (Sigma-Aldrich) in one half and buffer (0.1 to 0.8% DMSO) in the other half from the bottom of the abaxial side. At 32 to 36 HAI, leaf sections (~0.5 × 0.5 cm) were examined under a Zeiss Axioskop epifluorescence microscope for counting the number of haustoria labeled by RPW8.2-YFP and subsequently subject to trypan blue staining to visualize and estimate the total number of haustoria and induced cell death.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: RPW8 (AF273059), ADF5 (At2g16700), and ADF6 (At2g31200).

Author Contributions

W.W. performed most of the experiments with support from S.X., Y.W., and R.B.; S.X. and W.W. designed the experiments and wrote the manuscript.

Supplemental Data

The following materials are available in the online version of this article. Supplemental Figure 1. Two Distinct Patterns of RPW8.2-YFP Localization. Supplemental Figure 2. RPW8 Does Not Activate Penetration Resistance. Supplemental Figure 3. Plasma Membrane Damage/Repair Caused by Wounding Does Not Induce RPW8.2-YFP Expression and Plasma Membrane Localization. Supplemental Figure 4. A Cartoon Illustrating a Fungal Haustorium Developing into an Invaginated Epidermal Cell and the Targeting of RPW8.2 to the EHM.
Supplemental Figure 5. Targeting of RPW8.2 to the EHM Does Not Require SA Signaling and Is PEN1 Independent/Redundant.

Supplemental Figure 6. Targeting of RPW8.2 to the EHM Requires Function of the Actin Cytoskeleton.

Supplemental Figure 7. RPW8 Enhances the Formation of the Callosic Encasement of the Haustorial Complex and H₂O₂ Production.

Supplemental Figure 8. Polymorphisms in RPW8.2 Sequences and Disease Infection Phenotypes among Several Arabidopsis Accessions.

Supplemental Table 1. Primers Used for RT-PCR.

Supplemental Movie. EHM-Targeted Translocation of RPW8.2-YFP Vesicles.

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REFERENCES


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REFERENCES


Specific Targeting of the *Arabidopsis* Resistance Protein RPW8.2 to the Interfacial Membrane Encasing the Fungal Haustorium Renders Broad-Spectrum Resistance to Powdery Mildew

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