Expression of the Membrane-Associated Resistance Protein RPW8 Enhances Basal Defense Against Biotrophic Pathogens

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The powdery mildew resistance genes *RPW8.1* and *RPW8.2* from *Arabidopsis* differ from the other isolated plant resistance (*R*) genes in their predicted protein domains and their resistance spectrum. The two homologous *RPW8* genes encode small proteins featuring a predicted amino-terminal transmembrane anchor domain and a coiled-coil domain and confer resistance to a broad spectrum of powdery mildews. Here, we show that *Arabidopsis* plants expressing the *RPW8* genes have enhanced resistance to another biotrophic pathogen, *Hyaloperonospora parasitica*, raising the possibility that the *RPW8* genes may function to enhance salicylic-acid-dependent basal defenses, rather than as powdery-mildew-specific *R* genes. When overexpressed from their native promoters, the *RPW8* genes confer enhanced resistance to the *Cauliflower mosaic virus*, but render plants more susceptible to the necrotrophic fungal pathogens *Alternaria* and *Botrytis* spp. Furthermore, we show that the *RPW8* proteins appear to be localized to the endomembrane system, overlapping with the endoplasmic reticulum-associated small GTPase SAR1, and accumulate to higher levels in response to application of exogenous salicylic acid, one of the signaling molecules of plant defense.

Plants have sophisticated defense systems to protect themselves from different types of pathogens. Based on the mode of parasitism, plant pathogens generally can be divided into i) biotrophs that require live host cells at all stages of infection for completing their life cycle, ii) hemibiotrophs that require live host cells at an early stage of infection for successful colonization, and iii) necrotrophs that appear to kill host cells and feed on them (Glazebrook 2005). Tremendous progress has been made toward understanding the molecular mechanisms of plant resistance against biotrophic and hemibiotrophic pathogens. In many cases, resistance is controlled by resistance (*R*) proteins featuring a nucleotide binding site (NBS) and leucine-rich repeats (LRR) (Dangl and Jones 2001) and is associated with a hypersensitive response (HR), a rapid and localized cell death at the site of infection (Hammond-Kosack and Jones

1997; Heath 2000; Staskawicz et al. 1995). The NBS-LRR proteins are thought to directly or indirectly recognize pathogen effectors and activate local resistance at the site of infection and later systemic acquired resistance (SAR) in uninoculated parts of the plants through conserved signaling pathways that often engage the signaling molecule salicylic acid (SA) (Belkadir et al. 2004; Durrant and Dong 2004; Glazebrook 2001). In contrast, relatively little is known about how plants detect the invasion of necrotrophic pathogens and initiate defense signaling. However, many reports have demonstrated that plant hormones jasmonic acid (JA) and ethylene (ET) orchestrate the host defense signaling in response to attack from necrotrophic pathogens, and that there is cross-talk between the SA-dependent and JA-ET-dependent defense signaling (Feys and Parker 2000; Glazebrook 2005; Gupta et al. 2000). Generally, it has been shown that strong activation of one pathway represses the other, suggesting there is an overall mutually antagonistic relationship between these two distinct pathways although, at low magnitudes or early stages of signaling activation, these two pathways may be synergistic (Mur et al. 2006).

Powdery mildew comprises an important group of biotrophic fungal pathogens that cause diseases on numerous plant species (Braun 1987; Whipps et al. 1998). The two homologous *Arabidopsis* *R* genes, *RPW8.1* and *RPW8.2*, confer broad-spectrum resistance to multiple powdery mildew isolates that belong to distinct *Erysiphe* spp. (Xiao et al. 2001). In contrast, most characterized *R* genes confer resistance to one or a few isolates of a particular pathogen carrying the corresponding avirulence (*Avr*) gene (Hammond-Kosack and Jones 1997). Both *RPW8.1* and *RPW8.2* (together hereafter referred to as *RPW8*, unless otherwise indicated) encode small, basic proteins with a putative N-terminal transmembrane domain and a coiled-coil (CC) domain (Xiao et al. 2001). Our earlier analyses showed that transcription of the *RPW8* genes is induced to higher levels by powdery mildew; *RPW8*-overexpression from the native promoters results in spontaneous HR-like lesions (SHL) and induction of *PR* genes (Xiao et al. 2003); and the *RPW8* genes appear to trigger HR and resistance via a pathway that engages signaling components SA, *PAD4*, *EDS1*, *EDS5*, and *NPR1* (Xiao et al. 2005) that also are recruited by *NBS-LRR* genes containing a toll-interleukin receptor 1 (TIR)-encoding domain (Aarts et al. 1998; van der Biezen et al. 2002). Interestingly, these signaling components also are implicated in basal resistance against virulent biotrophic or hemibiotrophic pathogens (Wiermer et al. 2005; Xiao et al. 2005). A model has been proposed to explain *RPW8*-mediated broad-spectrum resistance (Xiao et al. 2005). In this model, expression of *RPW8* could be directly or indirectly induced by invasion of

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powdery mildew isolates and amplified by an SA-feedback circuit, leading to activation of defense responses via a conserved basal resistance pathway in a non-race-specific manner. Based on this hypothesis, it is anticipated that the *RPW8* genes may condition resistance against other types of biotrophic pathogen if appropriately induced at pathogen invasion sites. In this analysis, we show that expression of *RPW8* confers enhanced resistance to the oomycete *Hyaloperonospora parasitica*, a biotrophic pathogen different from powdery mildew. This provides direct evidence for the above model. However, overactivation of the SA pathway by *RPW8* overexpression leads to enhanced susceptibility to necrotrophic pathogens (*Alternaria* and *Botrytis* spp.), conforming to the notion of a mutually antagonistic relationship between SA- and JA-ET-dependent defenses. We also show that the *RPW8* proteins are associated mainly with the endomembrane system, overlapping with the small GTPase SAR1, and accumulate at higher levels upon exogenous SA application.

RESULTS

RPW8 expression leads to enhanced resistance to *H. parasitica*.

The *RPW8* locus contains two powdery mildew *R* genes, and transgenic plants with Col-0 containing the glabrous mutation, *gl-1* (Col-gl) expressing *RPW8.1* and *RPW8.2* from their native promoters did not show resistance to the oomycete pathogen, *H. parasitica* (*Hp*) isolate noco2 (Xiao et al. 2001). However, the disease test with *H. parasitica* noco2 was done

in 100% relative humidity (RH) and subsequent studies indicated that transcription of *RPW8.1* and *RPW8.2* was attenuated under these conditions (Xiao et al. 2003).

Therefore, we used two approaches to reexamine whether the *RPW8* locus contributes to resistance against *H. parasitica* noco2. The first was based on our observation that, although *RPW8* transcription was attenuated at 100% RH, it was not completely suppressed (Xiao et al. 2003). We reasoned that there may be sufficient *RPW8* transcription in lines containing multiple copies of *RPW8* to compensate for the reduced transcription at this humidity. The three homozygous Col-gl lines, S5, S6, and S24, containing a single copy, two copies, and at least four copies, respectively, of *RPW8* under control of the native promoters (Xiao et al. 2003) were tested, along with Col-gl and Ms-0 (the natural accession from which the two *RPW8* genes were isolated) (Xiao et al. 2001), for resistance to *H. parasitica* noco2. Seedlings were grown on Murashige-Skoog (MS)-agar medium for 2 weeks and then were transferred into sterilized perlite in Magenta boxes to permit *RPW8* transcription. Three days after transfer, plants were sprayed with noco2 and maintained inside perlite-containing magenta boxes (RH = 100%). Ms-0 plants were completely resistant to noco2 even under RH = 100% conditions (not shown), suggesting that Ms-0 may contain a functional *RPP5* (Parker et al. 1997) or similar *R* allele that confers resistance to the pathogen. S5 was only slightly less susceptible than Col-gl to *H. parasitica* noco2 (Fig. 1A[a] and B), in agreement with our previous results (Xiao et al. 2001). However, S6 and S24 plants had apparent enhanced resistance to noco2, with $\approx 3\times$

Fig. 1. *RPW8* expression enhances basal resistance to *Hyaloperonospora parasitica*. **A**, Seedlings (18 for each genotype) of indicated genotypes were grown in MS-agar medium and soil for 2 weeks and transplanted into perlite in sealed Magenta boxes (a) and into soil in pots (b and c), respectively, under relative humidity (RH) = 100% (a) and 85% (b and c). Three days after transplanting, the seedlings were inoculated with *H. parasitica* noco2 at a concentration of $\approx 5 \times 10^4$ spore/ml. Infection results were shown by representative leaves of each indicated genotype-treatment from the adaxial side (b) and abaxial side (c) at 6 days postinoculation (dpi). Induction of host cell death in inoculated leaves was revealed by trypan blue staining at 4 dpi (d); m = fungal mycelium and d = dead cells. **B**, Twenty most susceptible leaves from 18 seedlings of each genotype in A (a and b) were pooled, weighed, and counted for the number of spores per milligram of fresh tissue. These experiments were repeated twice with similar results and data from one of the three experiments were shown in B. **C**, Col-gl, S5, and S6 seedlings were prepared as in A(b) and infected with *H. parasitica* noco2 under RH = 100 and 85% conditions. Infected leaves were subject to transcript analysis at 4 dpi for *RPW8.1* and *PR1* by quantitative reverse-transcriptase polymerase chain reaction. The *RPW8.1* mRNA levels of all samples were compared with that in S5 under RH = 100% (1.00) and the *PR1* mRNA levels were compared with that in Col-gl under RH = 100% (1.00) using *ACT2* as the endogenous control (Xiao et al. 2003). Shaded data were calibrators used for comparison among relevant samples. This experiment was repeated once with similar results. Data from one experiment was shown.

and $\approx 19\times$ reduction, respectively, in the number of conidia produced per milligram of infected leaf tissue compared with Col-0 plants (Fig. 1A and B). We measured the mRNA levels of *RPW8.1* and *PR1* by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) in Ms-0, S5, S6, and S24 seedlings under 100% RH before inoculation. We found that the levels of mRNA for *RPW8.1* in S5, S6, and S24 were 1.1, 2.0, and 24.5 of that in Ms-0, respectively, and the levels of mRNA for *PR1* in S5, S6, and S24 were 1.0 $\times$, 2.4 $\times$, and 3.2 $\times$ of that in Ms-0, respectively, which generally agrees with the results obtained with soil-grown seedlings (Xiao et al. 2003) (discussed below and Fig. 1C). These observations indicated that higher levels of *RPW8* expression in S6 and S24 resulted in enhanced resistance to *H. parasitica* noco2.

The second approach was to keep the inoculated plants under the lowest possible RH that permitted infection by *H. parasitica* noco2, in the expectation that these conditions might minimize attenuation of *RPW8*-expression. We found that noco2 could not significantly infect Col-0 when RH was at or lower than 85%. However, when inoculated plants were kept under 100% RH for 24 h after inoculation and then maintained under $\approx 85\%$ RH for 5 to 7 days, Col-0 plants showed infection on inoculated leaves, with fewer conidiophores on the adaxial side of the inoculated leaves (Fig. 1A[b]) and more formed in the abaxial side (Fig. 1A[c]). We then tested soil-grown S5, S6, and Col-0 plants with the pathogen under the above conditions (100% RH for 1 day and 85% RH for 6 days). As expected, we found that S6 plants were nearly completely resistant to the pathogen. More significantly, S5 plants clearly had enhanced resistance to *H. parasitica* noco2, with an $\approx 3\times$ reduction in number of conidia produced per milligram of infected leaf tissue compared with Col-0 under these conditions (Fig. 1B). In most cases, enhanced resistance in the Col-0 *RPW8* transgenic lines appeared to be associated with pathogen-induced cell death, with S6 having the stronger and more rapid cell death (Fig. 1A[d]). To further establish the correlation between *RPW8* expression and enhanced resistance against noco2, we measured mRNA levels of *RPW8.1* and *PR1* by qRT-PCR in inoculated leaves of soil-grown Col-0, S5, and S6

seedlings at 4 days postinoculation (dpi). The *RPW8.1* mRNA levels were 2 to 3 $\times$ times higher in S6 than in S5 in both mock- and noco2-inoculated seedlings (Fig. 1C). This coincided with the slightly higher levels of *PR1* mRNA in both mock- and noco2-inoculated S6 in comparison with that of S5 (Fig. 1C) and higher level of resistance in S6 to the pathogen (Fig. 1A and B). Significantly, we found that, compared with mock inoculation, *RPW8.1* was induced by ≈ 3 and $\approx 2\times$ and *PR1* was induced by ≈ 1.5 and $\approx 2\times$ in S5 and S6, respectively, by *H. parasitica* noco2 at 85% RH. However, there was no apparent induction of *RPW8.1* or *PR1* in S5 or S6 by noco2 at 100% RH. To further confirm that it was the *RPW8* expression that rendered S5 with enhanced resistance to noco2, we tested plants containing the *RPW8* transgene from S5 and the *pad4-1* mutation under $\approx 85\%$ RH conditions and found that they were as susceptible as Col-0 (Fig. 1A[b and c]). *pad4-1* has been shown to greatly attenuate *RPW8* expression and abolish *RPW8*-mediated powdery mildew resistance (Xiao et al. 2005). Therefore, loss of enhanced resistance to noco2 in S5-*pad4-1* plants probably was caused by insufficient expression of *RPW8*.

Taken together, the above results indicate that *RPW8* expression enhances resistance to *H. parasitica* noco2, and suggest that *RPW8* may function in general defense rather than powdery-mildew-specific resistance.

***RPW8* overexpression leads to enhanced resistance to *Cauliflower mosaic virus*.**

To see whether *RPW8*-expressing plants have enhanced resistance to other types of pathogens, we inoculated *Cauliflower mosaic virus* (CaMV; Cabb B-JI strain) (Cecchini et al. 1998) on Col-0, S5, and S24 plants immediately after transfer from nonpermissive (MS agar + RH = 100%) to permissive conditions (perlite + RH = 85%). Both Col-0 and S5 (≈ 35 plants tested for each genotype) were fully susceptible to CaMV. Infected Col-0 and S5 plants developed typical vein-clearing or banding and chlorosis with curled leaf and distorted rosette at 15 to 20 dpi (Fig. 2A[a]), and both were unable to initiate inflorescence (Cecchini et al. 1998). However, 23 of 33 ($\approx 70\%$)

Fig. 2. *RPW8* overexpression results in altered responses to other pathogens. **A** (a through d), Five-week-old plants of the indicated genotypes grown in MS-agar plates were inoculated with *Cauliflower mosaic virus* strain Cabb B-JI (CaMV), *Pseudomonas syringae* pv. *maculicola* ES4326 (*Ps*), *Alternaria brassicicola* MUCL 20297 (*Ab*), and *Botrytis cinerea* IMI 169558 (*Bc*) and transplanted to perlite. In all, 15 to 35 plants for each genotype were used in each treatment. A representative leaf photographed at the indicated times was shown for each genotype–pathogen treatment. *Ab* and *Bc* fungi observed in the infection sites were shown as small inset pictures. These tests were repeated twice with similar results. **B**, Lesions caused by *Ab* and *Bc* in A were measured at 4 days postinoculation (dpi). Data represented the mean $\pm$ standard deviation from three independent experiments.

S24 plants had no disease symptoms, exhibited SHL, and developed normal inflorescence as mock-inoculated plants (not shown); the remaining 10 plants developed some degree of vein banding and chlorosis, which were less severe compared with Col-0 and S5 plants (data not shown). These results indicated that expression of *RPW8* at a very high level could lead to enhanced resistance against CaMV, which strictly requires live host cells to multiply.

***RPW8* overexpression has no effect on resistance to *Pseudomonas* spp. but enhances susceptibility to *Alternaria* and *Botrytis* spp.**

Five-week-old, MS agar-grown Col-gl, S5, and S24 plants were inoculated with the virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 and immediately transferred to perlite under conditions of RH = approximately 85%. Unexpectedly, we found no significant difference between Col-gl, S5, and S24 both in terms of disease symptoms (yellowing of inoculated leaves) and multiplication of bacteria (Fig. 2A[b]). However, there was an $\approx 2\times$ increase in bacterial growth and more rapid necrosis of inoculated leaves in S24 compared with Col-gl and S5. These results suggested that *RPW8* overexpression triggered SHL and activation of defense-related genes had no or slightly negative effect on defenses against *P. syringae* pv. *maculicola*.

Necrotrophic fungal pathogens have a mode of parasitism different from biotrophic pathogens (Glazebrook 2005). To analyze whether *RPW8* overexpression alters responses to this type of pathogens, we tested *Alternaria* and *Botrytis* spp., both of which are necrotrophic fungal pathogens that cause diseases in *Arabidopsis* (Glazebrook 2005). Preparation and growth

conditions of plants were the same as in the test with *P. syringae* pv. *maculicola*. Infection tests with *Alternaria brassicicola* (strain MUCL2029) showed that S24 was significantly more susceptible than Col-gl and S5, as indicated by progression of the pathogen around the inoculated sites and the diameter of the cell death lesions caused by the pathogen (Fig. 2A[c] and B). The pathogen-induced lesions on S5 were slightly bigger than those in Col-gl. Similar results were obtained with *Botrytis cinerea* (strain IMI169558), except that no difference was observed between Col-gl and S5 (Fig. 2A[d] and B). Wounding and mock inoculation did not induce any cell death on these three genotypes within the time frame of the tests. These results suggested that *RPW8* overexpression did not activate defenses against these necrotrophic pathogens; instead, the pathogens might have actively taken advantage of potentiation of HR cell death by *RPW8* overexpression in S24 to maximize their propagation.

***HA:RPW8.2* encodes a functional protein.**

The deduced *RPW8.1* and *RPW8.2* proteins contain an N-terminal transmembrane domain (*RPW8.1*, MPIGELAIGAVLG VGAQAIYDR; *RPW8.2*, MIAEVAAGGALGLALSVLHEAV; predicted by ConPred II and TMpred). For functional analysis of the *RPW8* proteins, we made a construct to produce a translational fusion between a haemagglutinin (HA) intron-epitope tag (Ferrando et al., 2000) and *RPW8.2* at the N terminus. The expression of the *HA-RPW8.2* fusion gene was controlled by the constitutive CaMV 35S promoter or the *RPW8.2* native promoter (*Np*) which is 1,000 bp upstream of the start codon of *RPW8.2* containing two W-boxes (Xiao et al. 2003). W-box (TTGACC/T) is a *cis*-acting element found in promoters of

Fig. 3. *HA:RPW8* is functional, enriched in membrane-enriched fractions, and induced by salicylic acid (SA). **A**, Four-week-old Col-gl wild-type and homozygous Col-gl transgenic plants expressing *HA-RPW8.2* under control of the native promoter (*Np*) were inoculated with *Erysiphe cichoracearum* UCSC1. Accumulation of H_2O_2 in plant cells and presence of fungal hyphae (h) and conidia (c) were revealed by 3,3'-diaminobenzidine and trypan blue staining, respectively (Xiao et al. 2003). Note that hypersensitive response lesions (arrowheads) associated with H_2O_2 production (indicated by brownish color) were visible in leaves of the transgenic lines (right) but were absent from wild-type Col-gl (left). Bars represent 100 μ m. **B**, Western blot analysis of protein extracts derived from leaves of a 4-week-old 35S::HA-RPW8.2 homozygous line. Samples were normalized for the amount of protein. Approximately equal amounts of total protein from soluble fraction and microsomal fractions were analyzed by immunoblotting. Correcting for total protein, $\approx 9\%$ of the protein was in S, whereas ≈ 22 and $\approx 69\%$ were associated with the 20,000 and 100,000 $\times$ g pellet fractions. **C**, Western blot analysis of protein extracts derived from leaves of a transgenic line containing an empty vector (V), the same 4-week-old 35S::HA-RPW8.2 homozygous line used in B (lane 1) and four homozygous lines of *Np::HA-RPW8.2* (lanes 2–5) sprayed with H_2O or 150 μ M SA. Total proteins were extracted from leaf tissues before or 48 h after treatment. Soluble fractions (s) and 16,000 $\times$ g pellets (p) were assayed. Approximately 50 μ g of total protein were loaded per lane and the HA:RPW8 fusion protein in the gel blot was detected with the monoclonal α -haemagglutinin antibody (Roche).

pathogen- or SA-responsive genes (Eulgem et al. 1999; Yu et al. 2001). These two constructs from the binary vector pBIN19-Plus were introduced into Col-gl background. The powdery-mildew-susceptible accession Col-0 lacks *RPW8.1* and *RPW8.2* (Xiao et al. 2001) and, thus, is ideal for functional test of the *HA-RPW8.2* fusion gene. Twenty T1 Col-gl transgenic plants were generated for each construct and tested with powdery mildew isolate *Erysiphe cichoracearum* UCSC1. In all, 15 *35S::HA-RPW8.2* T1 plants and 13 *Np::HA-RPW8.2* T1 plants

were moderately resistant to the fungus, with disease reaction score (DR) 1 or 1-2. HR lesions were visible on the inoculated leaves of those resistant T1 and T2 plants 4 to 5 dpi (Fig. 3A[a]). Diaminobenzidine (DAB) staining and subsequent trypan-blue staining (Xiao et al. 2003) were used to detect H_2O_2 accumulation induced by fungal penetration and to visualize the fungal structure. In inoculated Col-gl leaves, networks of fungal mycelia and conidiophores but no H_2O_2 -producing whole epidermal cells were observed (Fig. 3A[b]). In contrast,

in the inoculated leaves of resistant plants transgenic for either *35S::HA-RPW8.2* (not shown) or *Np::HA-RPW8.2*, there was H₂O₂ production in epidermal cells penetrated by the fungus and there was only limited fungal growth with no or few conidiophores (Fig. 3A[b]). These results indicated that the HA:RPW8.2 fusion protein is functional.

HA:RPW8.2 is enriched in the membrane fraction and is induced by SA.

Results obtained from preliminary Western blot analysis using anti-HA antibody suggested that levels of HA:RPW8.2 in the soluble fraction from independent transgenic lines were very low. It is possible that either the RPW8.2 protein was underrepresented in the total protein extract prepared with our protein extraction protocol, presumably due to its likely membrane association, or that it has a rapid turnover. To test whether RPW8.2 was associated with the membrane fraction, the total protein was extracted as described by Devoto and associates (2002). The cell homogenate was fractionated by differential centrifugation to separate membrane vesicles by mass and size, adapting a protocol from Devoto and associates (1999). Fractions were tested for the presence of HA:RPW8.2 by immunoblotting with an anti-HA antibody. HA:RPW8.2 was detectable only as a weak signal in the soluble fraction. The intensity of the signal increased in the microsomal fractions and particularly in the 100,000 × *g* high-speed pellet, at a mass of ≈22 kDa (Fig. 3B). These results suggest that the HA:RPW8.2 protein levels are higher in the membrane-enriched fraction.

In an earlier report, we showed that transcription of the *RPW8* genes under control of their native promoters involves a self-amplification mechanism via an SA-dependent feedback circuit (Xiao et al. 2003). However, it was not known whether enhanced transcription of the *RPW8* genes also led to enhanced level of the RPW8 proteins. To test this, independent transgenic lines homozygous for *HA-RPW8.2* driven by the native promoters or the 35S promoter were sprayed with 150 μM SA. After 48 h, leaves were harvested and total protein extracts were prepared. The soluble fraction and 16,000 × *g* pellet fraction were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and assayed by Western blot with anti-HA antibody. A line containing the *35S::HA-RPW8.2* transgene had higher levels of HA:RPW8.2 than in four *Np::HA-RPW8.2* lines (Fig. 3C). Significantly, there was no obvious increase in the HA:RPW8.2 level in the *35S::HA-RPW8.2* line after SA treatment. In contrast, the levels of HA:RPW8.2 in the four *Np::HA-RPW8.2* lines were lower before SA treatment and increased 3 to 10× in both the soluble and the membrane fractions after SA treatment. In addition, the intensity of the signal for HA:RPW8.2 was higher in the

membrane-enriched fraction than in the soluble fraction in all five transgenic lines (Fig. 3C). These results indicated that SA indeed enhanced the accumulation of the HA:RPW8.2 protein.

Expression and subcellular localization of fluorescence-tagged RPW8 proteins.

To determine the subcellular localization of the two RPW8 proteins, we prepared C-terminal translational fusions of the *RPW8.1* and *RPW8.2* coding sequences with the yellow fluorescent protein (YFP) so as to leave the putative N-terminus membrane localization signal intact. The sequence-confirmed chimeric genes *RPW8.1-YFP* and *RPW8.2-YFP* were cloned under control of the constitutive CaMV 35S promoter. These two constructs were introduced into onion epidermal cells by particle bombardment. Both RPW8.1:YFP and RPW8.2:YFP appeared to be localized mainly in the endomembrane system possibly including the plasma membrane, the endoplasmic reticulum (ER), and the Golgi apparatus, because a significant proportion of the RPW8:YFP fluorescence was displayed as punctate bodies of various sizes along the plasma membrane as well as in the cytoplasm (Supplementary Figure 1). Some fluorescent spots seemed to move along with the streaming strands. This punctate localization pattern of RPW8:YFP was in contrast to the homogeneous distribution pattern of YFP alone. We then transiently expressed the fusion proteins in leaf epidermal cells of *Arabidopsis* Col-gl plants by bombardment and observed punctuate patterns of fluorescence similar to those found in onion epidermal cells. Interestingly, we noticed that RPW8.1:YFP but not RPW8.2:YFP formed several big fluorescent punctate bodies proximal to chloroplasts (Fig. 4A; data not shown).

To analyze the subcellular localization of the RPW8 proteins, we compared their transient expression with that of two GFP-fusion proteins, the small GTPase SAR1 and the receptor of the ER-retention signal H/KDEL (ERD2), which are localized to ER export sites and Golgi body, respectively (daSilva et al. 2004; Hanton and Brandizzi 2006), along with the expression of YFP alone in *Arabidopsis* leaf epidermal cells (Fig. 4F). The punctate localization patterns of RPW8.1:YFP and RPW8.2:YFP were similar to those of SAR1 and ERD2, suggesting that the localization sites of the RPW8 proteins may include Golgi-ER-associated endomembranes.

To study the expression and localization of the RPW8 proteins in a more native state, we stably expressed *RPW8.1-YFP* and *RPW8.2-YFP* under control of their native promoters (discussed below) and examined leaves from at least five plants of 10 independent T2 transgenic lines for each construct by confocal microscopy. RPW8.1:YFP was detected as a fluorescent

Fig. 4. Subcellular localization and functional tests of fluorescence-tagged RPW8. Green fluorescent protein (GFP)- and yellow fluorescent protein (YFP)-tagged proteins were pseudocolored green, whereas DsRed were pseudocolored red and autofluorescent chloroplasts were pseudocolored A through F, red or H and I, blue. The z-axis distance between images of a stack and the number of images projected into a single plane are shown in brackets. All bars except the ones indicated represent 20 μm. Representative images of merged GFP channel and red channel in A–F showed localization of YFP tagged proteins or YFP alone in relation to chloroplasts. Note that the chloroplasts were viewed only in the red channel and YFP-tagged proteins were viewed only in the GFP channel. **A**, One representative *Arabidopsis* epidermal cell (EC) transiently expressing *Np::RPW8.1-YFP*. Arrows indicate bigger bright spots proximal to chloroplasts. **B**, *Arabidopsis* mesophyll cells (MC) (0.68 μm, 20 images) stably expressing *Np::RPW8.1-YFP*. **C**, An *Arabidopsis* midvein EC from the abaxial side (0.5 μm, 20 images) stably expressing *35S::RPW8.1-YFP*. **D**, An *Arabidopsis* EC stably expressing *Np::RPW8.2-YFP*. **E**, *Arabidopsis* MCs (0.64 μm, 20 images) stably expressing *Np::RPW8.2-YFP*. Arrow indicates a dead cell. **F**, An *Arabidopsis* EC transiently expressing *35S::YFP*. **G** and **H**, Sections of *Arabidopsis* midvein ECs from the abaxial side transiently coexpressing *35S::RPW8.1-DsRed* and *35S::SAR1-GFP* (G, single image; H, 1.4 μm, 10 images). Yellow-colored spots in G (indicated by arrowheads) and H reflect colocalization sites. **I**, Section of an *Arabidopsis* midvein EC from the abaxial side transiently coexpressing *35S::RPW8.2-DsRed* and *35S::SAR1-GFP*. **J**, Development of spontaneous hypersensitive response-like lesions (SHLs) in leaves of the indicated, representative transgenic lines. Arrows indicated SHLs. **K**, Disease reaction phenotypes of the indicated, representative transgenic lines at 8 days postinoculation of *Erysiphe cichoracearum* UCSC1. The expression of YFP alone was controlled by the 35S promoter, and the expression of *RPW8.1-YFP* or *RPW8.2-YFP* was controlled by their native promoters in J and K. Line S5 expressing both *RPW8.1* and *RPW8.2* by the native promoters was used as control in K.

ring-like structure surrounding or punctate spots proximal to chloroplasts in mesophyll cells (Fig. 4B) and punctate spots proximal to chloroplasts in epidermal cells in the midvein (Fig. 4C). *RPW8.1:YFP* was rarely detected in regular leaf epidermal cells. In contrast, *RPW8.2:YFP* was observed in both epidermal and mesophyll cells (Fig. 4D and E). Compared with *RPW8.1:YFP*, fluorescence from *RPW8.2:YFP* in mesophyll cells seemed less tightly associated with chloroplasts (Fig. 4E). It is important to note that, in both the *Np::RPW8.1-YFP* and *Np::RPW8.2-YFP* transgenic lines, YFP fluorescence was detected in only some leaf epidermal or mesophyll cells that were often but not always found in the proximity of a developing lesion (Fig. 4E). Development of SHLs in some of these transgenic lines was mostly likely due to overexpression of the functional fusion proteins (discussed below). These observations suggested that expression of *RPW8.1:YFP* and *RPW8.2:YFP* from their native promoters in leaf tissues is heterogeneous and may be spatially different from each other.

To further determine whether the two *RPW8* proteins localize to endomembranes associated with Golgi-ER, we tagged *RPW8* with DsRed (Jach 2006) and transiently coexpressed *35S::RPW8-DsRed* with *35S::SAR1-GFP* or *35S::ERD2-GFP* (daSilva et al. 2004) in the midvein epidermal cells of Col-gl leaves by bombardment. *SAR1:GFP* was detected as diffused fluorescence in the cytoplasm with or without the presence of bright punctate spots and ring-like structures (Fig. 4G-I), as previously reported (daSilva et al. 2004). The punctate accumulation of *SAR1:GFP* likely represents an ER export site, most of which moves along the surface of ER together with the Golgi bodies (daSilva et al. 2004). In the cells coexpressing *RPW8.1:DsRed* and *SAR1:GFP*, we observed that the punctate spots of *RPW8.1:DsRed* mostly colocalized with *SAR1:GFP* (Fig. 4G). However, as expected, *SAR1:GFP* displayed a wider range of distribution and those punctate structures where *RPW8.1:YFP* was not observed may represent Golgi bodies. Consistent with this speculation, coexpression indicated that *RPW8.1:DsRed* and *ERD2:GFP* did not exhibit colocalization. Because *RPW8.1* almost always localized proximal to chloroplasts, we collected signals from the red channel together with the GFP and DsRed channels to view the localization of *RPW8.1:DsRed*, *SAR1:GFP*, and chloroplasts. Indeed, *RPW8.1:DsRed* was found to accumulate around chloroplasts as various-sized fluorescent bodies where *SAR1:GFP* also accumulated, though the latter had a wider range of distribution (Fig. 4H). *RPW8.2:DsRed* also displayed overlapping localization with *SAR1:GFP* (Fig. 4I), but its association with chloroplasts was not as tight as *RPW8.1:DsRed* (Fig. 4E and I).

***RPW8.1:YFP* and *RPW8.2:YFP* were functional.**

To assess functionality of the *RPW8.1:YFP* and *RPW8.2:YFP* fusion proteins, we generated and phenotypically examined Col-gl transgenic lines expressing either one of these two fusion proteins under the control of their native promoters. We found that 17 of 118 *Np::RPW8.1-YFP* T1 lines and 35 of 120 *Np::RPW8.2-YFP* T1 lines developed SHL after plants were grown in short-day conditions for 3 weeks, whereas all *35S::YFP*-expressing plants did not (Fig. 4J), suggesting that these two fusion proteins are functional. Next, we inoculated plants of those transgenic lines with *E. cichoracearum* UCSC1. We found that $\approx 32\%$ of lines transgenic for *Np::RPW8.1-YFP* and $\approx 35\%$ of lines transgenic for *Np::RPW8.2-YFP* were resistant (disease score 0-1) or moderately resistant (1 or 1-2) (Xiao et al. 2005) to the pathogen (Fig. 4K). Furthermore, we tested 10 of the above powdery-mildew-resistant lines from each category (either *RPW8.1-YFP* or *RPW8.2-YFP*) with *H. parasitica* *noco2* under RH = 85% conditions as described in Figure 1A(b and c) and found that these lines exhibited various degrees of enhanced

resistance to *noco2* (data not shown), providing further evidence that *RPW8* expression confers enhanced resistance to *noco2*. Combined, these results indicated that both *RPW8.1:YFP* and *RPW8.2:YFP* fusion proteins are functional in activating SHL and (enhanced) resistance to biotrophic pathogens. Therefore, the endomembrane localization of the two *RPW8:YFP* fusion proteins observed in this analysis reflects the localization of the native *RPW8* proteins in *Arabidopsis* leaf tissues. In addition, this is consistent with the evidence from the localization of HA:*RPW8.2* based on SDS-PAGE fractionation and Western analysis (Fig. 3).

DISCUSSION

One of the most interesting features of *RPW8* is that it confers non-race specific resistance against powdery mildew (Xiao et al. 2001). Because *RPW8* involves a self-amplification mechanism via the SA-feedback circuit for activation of HR and resistance (Xiao et al. 2003, 2005), we hypothesized that *RPW8* engages the basal resistance machinery to potentiate defenses against powdery mildew pathogens and predicted that, if other biotrophic pathogens can induce *RPW8* expression, *RPW8* may trigger effective resistance against them (Xiao et al. 2005). Data from the present analysis provided strong evidence for this model. We showed that *RPW8* transcriptional amplification by SA-feedback circuit may lead to accumulation of the *RPW8* protein, because exogenous SA treatment increased the level of HA:*RPW8.2* under control of the native promoter (Fig. 3C). More importantly, we found that *RPW8* expression from a single copy of *RPW8* under control of the native promoters (line S5) confers enhanced resistance to *H. parasitica*, a biotrophic oomycete pathogen different from powdery mildew (Fig. 1A and B). There was no detectable elevated SA level and *PR-1* expression in soil-grown S5 plants compared with Col-gl plants (Xiao et al. 2003), suggesting that enhanced resistance to *H. parasitica* *noco2* is more likely due to *RPW8* expression induced by the pathogen (Fig. 1C). Most likely this enhanced resistance was underestimated because *RPW8*-expression was attenuated under the conditions of $\approx 85\%$ RH (Xiao et al. 2003) in which the tests were conducted. Indeed, higher levels of *RPW8* expressions due to the presence of two or more copies of the *RPW8* transgenes correlated with higher levels of enhanced resistance to *noco2* (Fig. 1A through C). Conversely, greater attenuation of *RPW8* expression by RH = 100% correlated with increased susceptibility of S5 and S6 to *noco2* (Fig. 1A through C). This is consistent with our previous observation that high-humidity conditions could compromise *RPW8*-mediated powdery mildew resistance in the same transgenic line S5 used in this analysis (Xiao et al. 2003), suggesting that the enhanced resistance of S5 to *noco2* most likely shares the same mechanism with that of powdery mildew resistance. These results collectively suggest that *RPW8* may be better defined as a signaling component that strengthens the SA-dependent signaling for basal resistance against biotrophic pathogens than as a powdery-mildew-specific *R* gene. When its expression is induced to a certain level, *RPW8* may initiate the SA-dependent feedback amplification circuit, resulting in sensitization for pathogen-induced HR cell death and thereby activating local resistance against biotrophic pathogens such as powdery mildews and oomycete *H. parasitica* in a non-race-specific manner. Higher *RPW8* expression also may lead to activation of SAR as indicated by higher levels of constitutive *PR1* expression in transgenic lines containing multiple copies of *RPW8* (Xiao et al. 2003, 2005; this analysis) and this also may contribute to enhanced resistance to *H. parasitica* in *RPW8*-overexpressing plants. A similar mechanism has been proposed for a newly cloned *R* gene, *RFO1*, which confers broad-spectrum resistance to *Fusarium* races (Diener and Ausubel 2005).

RFO1 is identical to the previously named *Arabidopsis* gene *WAKL22*, which encodes a receptor-like kinase that does not contain an extracellular LRR domain (Diener and Ausubel 2005). Like *RPW8*, *RFO1* also is induced by the pathogen and has been envisaged as a signaling component of a defense pathway (Diener and Ausubel 2005). Additional indirect evidence for *RPW8* as a signaling component is provided by the finding that *RPW8* is induced by *ssi4*, a gain-of-function mutation in a *TIR-NBS-LRR* gene that results in constitutive activation of defense responses (Zhou et al. 2004). Interestingly, *ssi4*-activated defenses also can be attenuated by high humidity (Shirano et al. 2002; Zhou et al. 2004). Testing *RPW8*-expressing plants with other types of biotrophic pathogens under normal RH (50 to 80%) conditions should help consolidate the conclusion that *RPW8* is better defined as a signaling gene that stimulates basal resistance. However, given that *Arabidopsis* accessions (such as Col-0) lacking *RPW8* can mount *R*-mediated resistance and basal resistance to different pathogens other than powdery mildew, *RPW8* is unlikely to be an indispensable component of plant basal resistance.

Plants of the *RPW8*-overexpressing line S24 also exhibited enhanced resistance to a viral pathogen, CaMV (Fig. 2A). Again, this is not unexpected; because the virus may strictly require live host cells for multiplication, SHL caused by *RPW8* overexpression may hinder spreading of the virus. Broad-spectrum resistance also has been observed in plants overexpressing other *R* genes (Oldroyd and Staskawicz 1998; Tang et al. 1999) or an *R*-signaling component (Cao et al. 1998; Chern et al. 2005). Interestingly, our data showed that *RPW8* overexpression did not have a significant effect on disease symptoms and growth of a virulent *P. syringae* strain. This suggests that basal resistance strengthened by *RPW8* overexpression is effective only in restricting biotrophic pathogens and that an additional or separate signal input is required for activation of basal resistance against hemibiotrophic bacterial pathogens. Similar phenotypes were reported for the *edr1* mutant, in which loss-of-function mutation in a *MAPKKK* gene confers enhanced resistance to powdery mildew but not to *P. syringae* (Frye and Innes 1998; Frye et al. 2001). In a recent report, we showed that *EDR1* appears to be a negative regulator of *RPW8* signaling (Xiao et al. 2005), which is consistent with a role of *RPW8* in stimulation of basal defenses specifically against biotrophic fungal pathogens. Also, we recently observed that the lesion-mimic mutant *acd5* with enhanced disease susceptibility to *P. syringae* pv. *maculicola* (Greenberg et al. 2000) displayed enhanced resistance to *E. cichoracearum* UCSC1 (S. Xiao, unpublished data), further supporting the notion that basal defense against strictly biotrophic pathogens may differ from that against hemibiotrophic bacterial pathogens such as *P. syringae*.

On the other hand, necrotrophic pathogens have a different mode of parasitism. Unlike biotrophic pathogens, they may not be restricted by host cell death. In fact, it was reported that HR induced by an avirulent *P. syringae* strain appeared to facilitate infection by *B. cinerea* (Govrin and Levine 2000). A plethora of evidence has now suggested that plant resistance to necrotrophic pathogens requires JA and ET signaling, which may actually be antagonistic to the SA-signaling pathway (Feys and Parker 2000; Glazebrook 2005; Gupta et al. 2000; Ton et al. 2002). Our observation that *RPW8*-overexpressing plants were more susceptible to necrotrophic fungal pathogens *A. brassicicola* and *B. cinerea* reinforces this notion. Weakened basal resistance against the two necrotrophs in the *RPW8*-overexpressing plants could be due to repression of the JA-ET signaling by hyperactivation of the SA signaling or possible facilitation of fungal growth by concurrent development of SHL. A detailed analysis of the interaction between SA signaling and JA-ET

signaling will be done to confirm this inference. Thus, although there is great potential in utilization of broad-spectrum *R* genes such as *RPW8* and *RFO1* or signaling components of the conserved defense pathways in controlling biotrophic pathogens, caution is required to avoid causing severe imbalance of different defense mechanisms when designing strategies for engineering plant disease resistance using specific *R* genes or *R* gene-signaling pathway components.

Determination of the subcellular localization of the *RPW8* proteins in the plant cell is the first step toward understanding how these proteins function in defense. The *RPW8.1* (GenBank accession number AAK09266) and *RPW8.2* (AAK09267) proteins are predicted to encode an N-terminal transmembrane domain or a signal peptide (by TMpred or TMHMM 2.0 and SignalP 3.0, respectively). By using a free web-based program, "Prediction of Protein Localization Sites" (PSORT), both *RPW8.1* and *RPW8.2* were predicted to be localized to ER membrane with a medium to a high probability (0.55 for *RPW8.1* and 0.82 for *RPW8.2*) and to plasma membrane (*RPW8.2*) and Golgi body (*RPW8.1*) with a low probability (0.19 and 0.10, respectively). Our results, showing that HA:*RPW8.2* was enriched in the membrane fraction (Fig. 3B and C) and *RPW8.1*:YFP and *RPW8.2*:YFP appeared to be localized to the endomembrane system, overlapping with the ER marker protein, the small GTPase SAR1 (Fig. 4G through I), generally agreed with the predictions by PSORT. Interestingly, we observed that, when expressed under the native promoters, *RPW8.1*:YFP was predominantly expressed in mesophyll cells whereas *RPW8.2*:YFP was expressed in both epidermal cells and mesophyll cells. This suggests that subtle differences in tissue-specific expression may exist between these two genes or proteins and there may be a synergistic or complementary effect in resistance when these two proteins are expressed together. This, in turn, may provide a likely explanation for evolution and maintenance of both *RPW8.1* and *RPW8.2* in a number of natural *Arabidopsis* accessions, including Ms-0, from which the two *RPW8* genes were isolated (Xiao et al. 2001). Considering that *RPW8.1* and *RPW8.2* were induced by powdery mildew (Xiao et al. 2005) and *H. parasitica* (this analysis), a relevant future question to be asked is whether and how the two *RPW8* proteins in the leaf epidermal and mesophyll cells change their expression levels or localization in response to pathogen penetration.

MATERIALS AND METHODS

Plant materials and cultivation.

Col-gl and T8 generation plants of three homozygous transgenic lines, S5, S6, and S24, containing a single copy, two copies, and at least four copies, respectively, of *RPW8.1* and *RPW8.2* under control of their native promoters (Xiao et al. 2003) were used for this analysis. Unless otherwise indicated, plants were grown on MS-agar medium under short-day conditions (8 h of light and 16 h of dark) for 2 to 5 weeks and subsequently transferred to perlite wetted with half-strength MS salt liquid in Magenta Vessels (77 by 77 mm, Sigma, St. Louis) and kept in long-day conditions (16 h of light and 8 h of dark) (Xiao et al. 2003). Light intensity was adjusted to $\approx 120 \mu\text{mol m}^{-2} \text{s}^{-2}$ in all the growth chambers. RH was controlled by covering the plants with plastic domes with or without holes, or magenta box lids with or without a 0.22- μm membrane vent.

Pathogen strains and inoculation.

Methods of inoculation for isolate *E. cichoracearum* UCSC1 were the same as reported (Xiao et al. 1997). Inoculation of *H. parasitica* noco2 isolate on 3- to 4-week-old plants

and quantification of disease susceptibility were conducted according to McDowell and associates (2000). For viral infection, *Brassica napus* leaves infected with CaMV (strain Cabb B-JI) were ground with 0.1 M Na₂PO₄ buffer and the supernatant then was filter (0.22 µm) sterilized. Inoculation was performed according to Cecchini and associates (1997). For bacterial infection, fully expanded leaves were infiltrated with a suspension of *P. syringae* pv. *maculicola* ES4326 ($\approx 10^4$ CFU/ml) in 10 mM MgCl₂. Leaf discs were sampled immediately after infiltration or at 4 dpi with a cork borer, ground in 10 mM MgCl₂, serially diluted, and plated on King's B agar plates. For infection with the necrotrophic pathogens *A. brassicicola* MUCL 20297 and *Botrytis cinerea* IMI 169558, leaves were punctured with a fine needle and applied with 2 µl of spore suspension (equivalent to 10⁸ spores/µl) or H₂O at the wound sites.

Generation of HA-RPW8.2 transgenic lines.

Coding sequences of *RPW8.2* (amplified by HAMC3F: 5'-CGGGATCCAATGATTGCTGAGGTTGCCGCA-3' and EcoR82R: 5'-CGGAATTCTCAAGAATCATCTGCAGAACGT-3') were in-frame cloned into *Bam*HI and *Eco*RI sites of plasmid pPILY which contains an intron-tagged HA epitope for translational fusion with HA at the N-terminus of *RPW8.2* (Ferrando et al. 2000). The recombinant plasmids, upon confirmation by sequencing, were digested with *Kpn*I to release the 2x35S::HA-*RPW8.2* cassettes which subsequently were cloned to the *Kpn*I site of the binary vector pBIN19-plus. For expression of HA-*RPW8.2* from its native promoter, a 1.0-kb 5' sequence upstream of ATG start codon of *RPW8.2* was amplified by 5'-CCCTAAGCTTACTCTCCAGCACT-3' and 5'-GCTCTAGATTTCTTGAAATTAGTTTGTAGCTC-3' and used to replace the 2x35S promoter sequence in the 2x35S::HA-*RPW8.2*/pPILY plasmid using the *Xho*I and *Hind*III sites. The cassette then was released by *Kpn*I digestion and cloned into the *Kpn*I site of pBIN19-plus. The recombinant pBIN19-plus plasmids were introduced into *Agrobacterium* GV3101 and used for transformation of Col-gl plants.

Transient and stable expression of fluorescence-tagged *RPW8* proteins.

To make an YFP-tagged *RPW8* gene, *RPW8.1* and *RPW8.2* 3' flanking sequences were amplified by BamR813'F (5'-GAGGATCCAGAGCTTGATAAAAAACAGAG-3') and BglR813'R (5'-ACATAGATCTCAAAAGAAGCGAA-3') and BamR823'F (5'-ATGGATCCTGATGTCCTAATTGTGTCAAAC-3') and BglR823'R (5'-TGAGATCTTTTGTGTTTCTTACTCT-3'), respectively, cut by *Bam*HI and *Bgl*II, and cloned into the *Bam*HI site of binary vector pPZP211 (Hajdukiewicz et al. 1994), resulting in plasmids pPZPR813' and pPZPR823', respectively. The YFP coding region was amplified from pEYFP-C1 (Clontech, Palo Alto, CA, U.S.A.) with primers 5'-TCGGA TCCATGGTGAGCAAGGGCGAG-3' and 5'-GCAGATCTCA CTTGTACAGCTCGTCCATG-3', digested with *Bam*HI and *Bgl*II, and cloned into the *Bam*HI site of pPZPR813', pPZPR823', and binary vector pCB302-3 (Xiang et al. 1999), resulting in plasmids pPZPYFP13', pPZPYFP23', and pBamEYFP. For constitutive expression of *RPW8*-EYFP fusion protein, the *RPW8.1* coding sequence (amplified with primers HAMC2F 5'-CGGGATCCAATGCCGATTGGTGAGCTTGC GATA-3' and BamR81R 5'-GCGGATCCAGCTCTTATTTTA CTACAAGCAGA-3') and *RPW8.2* coding sequence (amplified with primers HAMC3F and BamR82R 5'-CGCGATCCAGA ATCATCACTGCAGAACGTAAA-3') were in-frame cloned into the *Bam*HI site of pBamEYFP, resulting in plasmids pR81EYFP and pR82EYFP, respectively. For tests of *RPW8* colocalization with SAR1:GFP and ERD2:GFP, YFP coding sequences in pR81EYFP and pR82EYFP were replaced by

DsRed coding sequences amplified from pDsRed-Express (Clontech) with primers DsRedF (5'-TAGGATCCATGGCCT CCTCCGAGG-3') and DsRedR (5'-AAAGATCTCCGCTACA GGAACAGGTGGTG-3'), resulting in plasmids pR81DsRed and pR82DsRed. The plasmid DNAs of pR81EYFP and pR82EYFP were introduced into onion epidermal cells or Col-gl leaves, and plasmid DNAs of pR81DsRed or pR82DsRed were introduced into Col-gl leaves together with plasmid DNA containing 35S::SAR1-GFP or 35S::ERD2-GFP by using a Biolistic PDS-1000/He system (BioRad, Hercules, CA, U.S.A.) following the manufacturer's instruction. Tungsten particles (M-20) were coated with the respective plasmid DNA and a helium pressure of 7.5 MPa (1,100-psi rupture) was employed. The target distance between the stop screen and onion piece or Col-gl leaves was set at 6 cm. After bombardment, onion pieces or Col-gl leaves were kept in darkness at room temperature for 15 to 24 h before examination under a confocal microscope. For native expression of the *RPW8*:YFP fusion proteins, the promoter and coding sequences of *RPW8.1* and *RPW8.2* were amplified with BamR81PF (5'-TCGGATCCATTGGTCTCTCA ATTTGTTTATT-3') and BamR81R, BamR82PF (5'-TTGGAT CCTCACCGAAATTGTTAGTATTCACG-3') and BamR82R, cut by *Bam*HI, and cloned into the *Bam*HI sites of pPZPYFP13' and pPZPYFP23', generating plasmids pPR81EYFP and pPR82EYFP, respectively. Then, pPR81EYFP and pPR82EYFP were introduced into Col-0 and Col-gl by *Agrobacterium*-mediated transformation. All T1 transformants were examined for development of SHL, disease resistance to powdery mildew isolate *E. cichoracearum* UCSC1. Transient or stable expression of YFP or *RPW8*-YFP fusion proteins in T2 transgenic plants were examined and imaged using a Zeiss LSM 510 laser scanning confocal microscope with $\times 10$ NA 0.8 dry and $\times 63$ NA 1.2 water-immersion lenses (Carl Zeiss Inc., Thornwood, NY, U.S.A.). Single optical sections or a stack of z sections were obtained by adjusting pinhole, detector gain, and laser transmission according to the user's manual of Zeiss LSM510 to produce the best signal/noise ratio. GFP or YFP fluorescence was acquired with an Argon 488-nm laser exciter and a band pass emission filter BP 505-550 (GFP channel). Auto fluorescence of the chloroplast was acquired with the same exciter and a long-pass emission filter LP 650 (red channel). For colocalization experiment of *RPW8*:DsRed with SAR1:GFP or ERD2:GFP, multi-tracks were configured with the GFP channel setting as above plus a DsRed channel with an HeNe1 543-nm laser exciter and a band pass emission filter BP 560-615. The image data were processed using Adobe Photoshop (Mountain View, CA, U.S.A.).

Protein extraction, membrane vesicle fractionation, and Western blot analysis.

The total protein was extracted as described by Devoto and associates (2002). For differential centrifugation experiments, we used a protocol adapted from Devoto and associates (1999). The cell homogenate was centrifuged at 3,000 $\times g$ for 5 min at 4°C. The supernatant was recovered and further centrifuged at 10,000 and 20,000 $\times g$ for 20 min and then at 100,000 $\times g$ for 45 min, with the supernatant from each spin taken for use in the next centrifugation step. The resulting pellets following each centrifugation step were resuspended in cold lysis buffer. Proteins were quantified according to the method of Bradford using bovine serum albumin as a standard. Protein extracts were incubated in SDS denaturing buffer (200 mM Tris-HCl, 20% [wt/vol] glycerol, 5 mM EDTA, 0.02% [wt/vol] bromophenol blue, and 2% [wt/vol] SDS) for 5 min at 70°C, separated by 15% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Roche, Branchburg, NJ, U.S.A.). To detect HA:RPW8, we used anti-HA antibody

(Roche). Blots were developed using the SuperSignal West Femto maximum sensitivity substrate (Pierce, Rockford, IL, U.S.A.).

Other analyses.

Methods for quantification of relative mRNA levels of *RPW8.1* and *PR1* by Applied Biosystems 7300 Real-Time PCR System were the same as previously described (Xiao et al. 2003). Histochemical detection of H₂O₂ with 3,3'-diaminobenzidine and host cell death and fungal structure by trypan blue also were according to Xiao and associates (2003).

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