Homologues of the RPW8 Resistance Protein Are Localized to the Extrahaustorial Membrane that Is Likely Synthesized De Novo1[OPEN]

Robert Berkey, Yi Zhang2, Xianfeng Ma, Harlan King, Qiong Zhang, Wenming Wang, and Shunyuan Xiao*

Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, Maryland (R.B., Y.Z., X.M., H.K., Q.Z., S.X.); The Rice Research Institute, Sichuan Agricultural University, Chengdu 611130, China (W.W.); and Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park, Maryland (S.X.)

ORCID IDs: 0000-0002-2988-778X (R.B.); 0000-0002-1566-098X (Y.Z.); 0000-0003-1348-4879 (S.X.)

Upon penetration of the host cell wall, the powdery mildew fungus develops a feeding structure named the haustorium in the invaded host cell. Concomitant with haustorium biogenesis, the extrahaustorial membrane (EHM) is formed to separate the haustorium from the host cell cytoplasm. The Arabidopsis resistance protein RPW8.2 is specifically targeted to the EHM where it activates haustorium-targeted resistance against powdery mildew. RPW8.2 belongs to a small family with six members in Arabidopsis (Arabidopsis thaliana). Whether Homologs of RPW8 (HR) 1 to HR4 are also localized to the EHM and contribute to resistance has not been determined. Here, we report that overexpression of HR1, HR2, or HR3 led to enhanced resistance to powdery mildew, while genetic depletion of HR2 or HR3 resulted in enhanced susceptibility, indicating that these RPW8 homologs contribute to basal resistance. Interestingly, we found that N-terminally YFP-tagged HR1 to HR3 are also EHM-localized. This suggests that EHM-targeting is an ancestral feature of the RPW8 family. Indeed, two RPW8 homologs from Brassica oleracea tested also exhibit EHM-localization. Domain swapping analysis between HR3 and RPW8.2 suggests that sequence diversification in the N-terminal 146 amino acids of RPW8.2 probably functionally distinguishes it from other family members. Moreover, we found that N-terminally YFP-tagged HR3 is also localized to the plasma membrane and the fungal penetration site (the papilla) in addition to the EHM. Using this unique feature of YFP-HR3, we obtained preliminary evidence to suggest that the EHM is unlikely derived from invagination of the plasma membrane, rather it may be mainly synthesized de novo.

1 This project was supported by National Science Foundation grants no. IOS-1146589 and IOS-1457033 to S.X.
2 Present address: Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850.
* Address correspondence to xiao@umd.edu.

The responsible author for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Shunyuan Xiao (xiao@umd.edu).

R.B. did most of the genetic and localization analyses; Y.Z. did the work concerning the origin of the EHM; X.M. determined the localization of HR4; H.K. isolated haustorial complexes with YFP-HR3-labeled EHM; Q.Z. helped R.B generation of transgenic lines; W.W. participated in experimental design; R.B. and S.X. designed the experiments and wrote the manuscript.

[OPEN] Articles can be viewed without a subscription. www.plantphysiol.org/cgi/doi/10.1104/pp.16.01539

Copyright © 2017 American Society of Plant Biologists. All rights reserved.
interfacial membrane termed the extrahaustorial matrix (EHM; Gil and Gay, 1977; Heath, 1976; Gil and Gay, 1977; Roberts et al., 1993). Together with the extrahaustorial matrix, the EHM represents the host-pathogen interface where fungal effectors are translocated into the host cell, and nutrients from the host cell are transported into the haustorium. Concomitant with the biogenesis of the EHM, a physical barrier named the neckband is thought to form and seal the fungal plasma membrane (PM) in the haustorial neck with the invaginated host PM to prevent the apoplastic escape of materials from the extrahaustorial matrix (Heath, 1976; Gil and Gay, 1977).

In response to haustorial invasion, plants mount postpenetration resistance to constrain the haustorium or limit its function. At the cellular or subcellular level, postpenetration resistance is manifested by the formation of a haustorial encasement and focal accumulation of H$_2$O$_2$ around the haustorium, with occasional collapse of the invaded cell (i.e. hypersensitive response; Meyer et al., 2009; Wang et al., 2009; Wen et al., 2011). Both the papilla and the haustorial encasement are enriched for callose (β-1,3-glucan) and these two structures appear to be spatiotemporally connected (Meyer et al., 2009). At the molecular level, apart from resistance activated by immune receptors upon recognition of specific pathogen effectors (Hallerman et al., 2001; Zhou et al., 2001; Yahiaoui et al., 2004; Jordan et al., 2011), plants have also evolved other forms of resistance mechanisms. For example, the Arabidopsis (Arabidopsis thaliana) atypical R proteins RPW8.1 and RPW8.2 confer postpenetration resistance to all infectious powdery mildew strains tested (Xiao et al., 2001). RPW8.1 and RPW8.2 belong to a small gene family in Arabidopsis and Brassica with the progenitor being a HOMOLOG of RPW8 3 (HR3)-like gene (Xiao et al., 2004). We recently revealed that RPW8.2 [also RPW8.1 when expressed in the haustorium-invaded cells (Ma et al., 2014)] is specifically targeted to the EHM where it enhances formation of the haustorial encasement and promotes on site H$_2$O$_2$ accumulation and/or triggers hypersensitive response (Wang et al., 2009). Specific targeting of RPW8.2 to the EHM requires two basic residue-enriched motifs in RPW8.2 and engages an EHM-oriented specific trafficking pathway (Wang et al., 2013; Kim et al., 2014). Thus, the EHM is the subcellular locale for launching RPW8.2-mediated, haustorium-targeted defenses. These observations demonstrate that the EHM is not only a site for cross-border trafficking but also a critical host-pathogen battleground. Whether other members of the RPW8 family play roles in postpenetration resistance and/or are localized to the EHM have not been determined.

In this study, through genetic and localization analyses we showed that three Arabidopsis RPW8 homologs (AtHR1 to AtHR3) and two representative Brassica oleracea RPW8 homologs (BoHRa and BoHRb) are probable EHM resident proteins and all but HR4 likely contribute to basal resistance against powdery mildew pathogens. Unexpectedly, we found that HR3 tagged with YFP at its N terminus is also localized to the PM and the papilla. Using the triple localization feature of YFP-HR3, we obtained preliminary evidence to suggest that the EHM is unlikely derived from invagination of the PM, rather it may be mainly synthesized de novo.

RESULTS

RPW8 Homologs Contribute to Basal Resistance against Powdery Mildew

The RPW8 gene locus in Arabidopsis Ms-0 accession contains RPW8.1 and RPW8.2 and three homologs of RPW8, designated HR1 (At3g50450), HR2 (At3g50460), and HR3 (At3g50470; Xiao et al., 2001). The powdery mildew-susceptible accession Col-0 lacks RPW8.1 and RPW8.2, but contains HR4 (At3g50480) in the same location, along with HR1, HR2, and HR3 (Xiao et al., 2001, 2004). Because Col-0 mutants including those that are defective in salicylic acid (SA)-signaling display enhanced disease susceptibility to powdery mildew (Xiao et al., 2005), we reasoned that Col-0 is still capable of mounting a certain level of SA-dependent and perhaps SA-independent basal resistance. To test if HR1 to HR4 play a role in basal resistance in Col-0, we first overexpressed HR1, HR2, HR3, and HR4 using the 35S promoter together with the native 5′ regulatory sequence (496 bp for HR3, 1000 bp for the rest), a demonstrated strategy shown to effectively achieve higher expression of RPW8.1 and RPW8.2 (Orgil et al., 2007), in Col-gl (Col-0 containing the glabrous mutation gl1-1). For each DNA construct, >30 T1 transgenic plants were generated and five independent homozygous lines in the T3 or T4 generation were tested with a well-adapted powdery mildew isolate Golovinomyces cichoracearum (Gc) UCSC1. Plants of all tested lines transgenic for 35S::HR1, 35S::HR2, or 35S::HR3 displayed obvious enhanced diseases resistance compared to Col-gl, whereas plants transgenic for 35S::HR4 were as susceptible as Col-gl (Fig. 1, A and C). Reverse-transcription (RT)-PCR confirmed that expression levels of the four RPW8 homologs were much higher in the transgenic lines than the respective endogenous genes in Col-gl (Fig. 1B). These results suggest that HR1, HR2, and HR3, but not HR4, may contribute to basal resistance in Col-0 against powdery mildew.

Next, we identified one T-DNA knockdown (kd) line (Salk_056764) for HR1 (designated HR1kd1), one knockout (ko) line (Salk_093095) for HR2 (designated HR2ko1) and two knockout lines (SALK_122954 and WiscDsLox420C08) for HR3 (designated HR3ko1 and HR3ko2) based on RT-PCR results (Fig. 1D and Supplemental Fig. S1). Plants of these mutant lines were inoculated with Gc UCSC1 along with plants of Col-0 and Col-NahG, a transgenic line expressing a bacterial SA hydrolase (Lawton et al., 1995). Except for HR1kd1, the remaining three T-DNA lines exhibited a small but reproducible increase in fungal spores on the leaf surface 10-12 d postinoculation (dpi) when compared to Col-0, although they were not as susceptible as Col-NahG (Fig. 1, E and F). Disease quantification
showed that plants of the HR2 and HR3 mutant lines produced approximately 25% to approximately 45% more fungal spores than Col-0 while Col-NahG supported nearly twice as many spores as Col-0 (Fig. 1F) at 12 dpi, indicating that genetic depletion of HR2 and HR3 results in enhanced disease susceptibility albeit at a lower degree compared to SA depletion by the SA hydrolase encoded by NahG. These results, together
with the data from overexpression analyses, demonstrate that HR1, HR2, and HR3 probably contribute to basal resistance against powdery mildew.

**Functional Diversification between RPW8.2 and HR3**

In a recent study, we found that two R/K-R/K-X-R/K motifs comprise the core EHM-targeting signal (ETS) in RPW8.2 (Wang et al., 2013). Sequence analysis showed that while the first ETS has some variations, the second ETS is highly conserved among all the RPW8 family members in Arabidopsis and three RPW8 homologs from *B. oleracea* (Supplemental Fig. S2). Thus, we wondered if other Arabidopsis RPW8 family members are also EHM-resident proteins. RPW8.1-YFP has shown to be mainly expressed in mesophyll cells when expressed from its native promoter [NP; (Wang et al., 2007)]. Interestingly, when expressed from the RPW8.2 promoter, RPW8.1-YFP was also found to be EHM-localized (Ma et al., 2014). To examine subcellular localization of HR1 to HR4, each of these genes was translationally fused with YFP at the C terminus, and the fusion constructs were stably expressed in Col-gl by their respective NPs. Unexpectedly, we were unable to detect any fluorescent signal before or after inoculation with *Gc UCSC1* from at least 30 independent T1 transgenic lines examined for each construct despite the detection of the transgene expression by RT-PCR in two representative lines per construct (not shown). Western blotting using an anti-GFP antibody recognizing YFP also revealed no protein accumulation in any of these lines (Supplemental Fig. S3A). While T1 plants transgenic for NP::HR1-YFP, NP::HR2-YFP, or NP::HR4-YFP appeared phenotypically normal in growth and development and were susceptible to powdery mildew (Fig. 2A), approximately 55% of T1 plants transgenic for NP::HR3-YFP exhibited stunted growth with leaf necrosis and varied degree of enhanced resistance to Gc UCSC1 (Fig. 2, B and C). These observations suggest that these fusion proteins cannot accumulate to levels detectable by confocal microscopy or western blotting, and that HR3-YFP is also toxic when stably expressed in Arabidopsis. To make sure that these fusion constructs are translated into proteins, we transiently expressed them in *Nicotiana benthamiana* leaves by agroinfiltration. Again, no YFP signal was detectable. We then transiently coexpressed each of the constructs with the coat protein of *Turnip crinkle virus* that also functions to suppress RNA silencing in host plants (Qu et al., 2003). Interestingly, except for NP::HR4-YFP, the remaining three constructs produced detectable, albeit weak, YFP signal. HR1-YFP and HR3-YFP appeared to be localized in the PM in epidermal cells, punctate spots were also present.
seen around chloroplasts in mesophyll cells. However, HR2-YFP was mainly found in the ring structures surrounding chloroplasts in mesophyll cells (Supplemental Fig. S3B), which is reminiscent of RPW8.1-YFP’s localization in mesophyll cells (Wang et al., 2009). Moreover, consistent with cell death in Arabidopsis lines transgenic for NP::HR3-YFP, we found massive necrosis from the leaf section infiltrated with NP::HR3-YFP only (Supplemental Fig. S3C), demonstrating that HR3-YFP is capable of triggering cell death in both Arabidopsis and *N. benthamiana*. Taken together, these observations suggest that RPW8 homologs appear to be distinct from RPW8.2 in terms of protein function and accumulation.

Given that an HR3-like gene is the probable progenitor of the RPW8 gene family (Xiao et al., 2004), we investigated how RPW8.2 might have functionally diversified from HR3 via the following three aspects. First, we expressed HR3-YFP from the RPW8.2 promoter (R82p) in the Col-0 background. We found that approximately 86% (42 of 49) of transgenic T1 plants showed strong or lethal cell death (Fig. 2D), suggesting that the RPW8.2 promoter is stronger than that of HR3. Again, YFP signal was not detected in any of these transgenic lines even before onset of cell death. Second, because substitution of Asp at 116 to Gly (D116G) in RPW8.2 abolishes RPW8.2-triggered hypersensitive response and resistance (Orgil et al., 2007; Wang et al., 2009) and D116 is conserved in HR3 (Fig. 3A), we thus made the D116G mutation in HR3 and stably expressed the HR3D116G-YFP fusion protein from the HR3 promoter. We found that this mutation did not affect HR3-YFP-triggered cell death (Fig. 2D) despite that the fusion protein remained undetectable. This suggests that hypersensitive response cell death activated by RPW8.2 is mechanistically distinct from the necrotic cell death caused by HR3-YFP. Third, because both RPW8.1 and RPW8.2 contain a shorter C terminus compared to HR3 (Xiao et al., 2004) and the C terminus SDDS of RPW8.2 appears to be required or full scale interaction with 14-3-3 (Yang et al., 2009), we made an HR3 variant with a truncation of the C-terminal 26 amino acids (ΔCt26) and the G186 to D substitution to make an SDDS C terminus as seen in RPW8.2 such that the resultant HR3ΔCt26/G186D mutant most structurally resembles RPW8.2 (Fig. 3A). Also, in another construct we removed the C-terminal 65 amino acids of HR3 (HR3ΔCt65) to see if it can make the fusion protein more stable. However, in both cases, cell death still occurred in respective T1 transgenic plants without detectable YFP signal (Fig. 2D). Based on these results we reasoned that the N-terminal portion of HR3 (amino acid 1 to 147) is responsible for (1) inappropriate cell death activated by HR3-YFP and (2) lack of protein accumulation at a detectable level by microscopy when the C-terminal portion is perturbed (i.e. due to fusion with a fluorescent protein). We speculated that these properties of HR3 might have been lost in RPW8.2 as part of its functional diversification from an HR3-like progenitor.

To further test the above speculation, we made chimeric constructs by swapping the N- and C-terminal
domains of RPW8.2 and HR3, which are designated R82n (amino acids 1 to 146)-HR3c (148 to 213), and HR3n (1 to 147)-R82c (147–174) as shown in Figure 3, A and B. Interestingly, R82n-HR3c-YFP was nicely localized to the EHM (Fig. 3C) and conferred resistance to Gc UCSC1 (Fig. 3D) in a similar manner as RPW8.2-YFP, whereas HR3n-R82c-YFP was still undetectable and lost ability to trigger cell death or resistance. These results suggest that (1) R82n is essential for protein stability, defense and EHM-localization of RPW8.2 while R82c is replaceable, which is compatible with results from our recent mutational analysis of RPW8.2 (Wang et al., 2009); (2) R82c may be able to suppress HR3n-mediated inappropriate cell death and/or that HR3n-R82c-YFP cannot accumulate to a level sufficient for triggering cell death. However, a more detailed structure-function analysis between HR3 and RPW8.2 is needed to further define the molecular and evolutionary mechanisms underlying the functional diversification between these two proteins.

Because HR3 has more likely retained the original conserved function of the HR3-like progenitor gene (Xiao et al., 2004), whereas RPW8.2 has apparently evolved novel resistance function, we also wondered if HR3 is required for RPW8.2-mediated resistance. To test this, we introduced the HR3-ko1 allele to S5, a Col-gl transgenic for a single copy of a genomic fragment containing RPW8.1 and RPW8.2 and their NPs by crossing. We found that S5/RHR3-ko1 plants were as resistant as S5 plants (Supplemental Fig. S4), indicating that HR3 is dispensable for RPW8.2-activated resistance.

All RPW8 Homologs Are Likely EHM-Resident Proteins

From the above analysis, we were unable to determine if the RPW8 homologs are also EHM-resident proteins due to the unknown intrinsic constraints associated with C-terminal YFP tagging. Neither did we succeed in detection of internally YFP-tagged HR3 by microscopy (not shown). Previously we reported that N-terminally YFP-tagged RPW8.2 (YFP-RPW8.2) was also targeted to the EHM, despite its loss of function in defense (Wang et al., 2010). We thus made N-terminally YFP-tagged DNA constructs for HR1, HR2, HR3, and HR4 and stably expressed them from the 35S promoter in Col-gl. While YFP signal was still not detectable from any of over 40 transgenic lines expressing YFP-HR4, YFP signal from the remaining three fusion constructs appeared to be distributed in the cytoplasm and possibly in the PM (Fig. 3A). To see if the YFP-HR4 fusion protein can be made, we transiently expressed the construct in N. benthamiana leaves and detected YFP signal along the cell wall possibly in the PM (Fig. 4A). These results suggest that N-terminally YFP-tagged HR4 is unable to accumulate in stable transgenic Arabidopsis plants, which is different from those of the other family members. To determine if the observed YFP signal from YFP-HR3 was indeed in the PM, we transiently coexpressed YFP-HR3 with an Arabidopsis PM aquaporin AtPIP2A in fusion with mCherry (Nelson et al., 2007) in N. benthamiana leaf cells. We found that YFP signal nicely colocalized with mCherry signal (Fig. 4B), indicating that N-terminally YFP-HR3 is localized to the PM.

We then inoculated respective transgenic lines with Gc UCSC1 to examine the localization of these fusion proteins in epidermal cells invaded by haustoria. Interestingly, we found that YFP-HR1, YFP-HR2, YFP-HR3 appeared to be preferentially or exclusively localized to the EHM in epidermal cells invaded by haustoria (Fig. 4C), similar to our observation with YFP-RPW8.2 (Wang et al., 2010). The EHM-localization of YFP-HR3 is confirmed by precise colocalization with RPW8.2-RFP at the EHM (Fig. 5A). Moreover, YFP-HR3 signal

Figure 4. Subcellular localization of YFP-HR1, YFP-HR2, YFP-HR3, and YFP-HR4. Except for YFP-HR4, which was transiently expressed in leaves of N. benthamiana, the rest were stably expressed in Arabidopsis Col-0 (At Col-0). A, Representative images showing cytoplasmic and likely plasma membrane localization of indicated fusion proteins expressed from the 35S promoter in leaves of Arabidopsis transgenic plants (for HR1, HR2 and HR3) or agroinfiltrated N. benthamiana plants (for HR4). B, A representative image showing colocalization of YFP-HR3 with AtPIP2A-mCherry in N. benthamiana epidermal cells when transiently coexpressed. C, Representative images showing likely EHM-localization of indicated fusion proteins expressed from the 35S promoter at 2 dpi with Gc UCSC1. D, EHM-localization of YFP-HR3 expressed from the HR3 NP in At Col-0 at 2 dpi with Gc UCSC1. Bar, 20 μm in A; 50 μm in B; 10 μm in C and D. H, Haustorium; Nb, N. benthamiana.
to the EHM, we inoculated YFP-HR4 expressing the EHM (Fig. 4D). To test if YFP-HR4 is also localized and YFP signals were mostly or exclusively found in transgenic lines expressing YFP-HR3 from the expression of the fusion protein, we also generated inability that EHM-localization was caused by over-

Figure 5. YFP-HR3 is localized to the plasma membrane, the EHM, and the papilla. A, Representative image showing that YFP-HR3 is localized to the PM (arrow), the EHM labeled by RPW8.2-RFP, and the papilla (arrowhead). Inset is a close-up view of the transverse section of a fungal penetration site where both YFP-HR3 and callose focally accumulate. Note the heterogeneous distribution of YFP-HR3. B, Representative isolated haustorial complex with an intact EHM labeled by both YFP-HR3 and RPW8.2-RFP. C, PM-localization of GFP-SYP131 in the epidermal cells containing haustoria (arrows). D, Maximum projection of a Z-stack image showing YFP-RPW8.2 is localized to the EHM but not in the papilla where callose is deposited (arrow). Bar, 10 μm in A, B, and D; 50 μm in C. Cp, Chloroplast.

remained detectable at the EHM of isolated haustorial complexes extracted from infected leaves coexpressing YFP-HR3 and R82-RFP (Fig. 5B). To exclude the possibility that EHM-localization was caused by over-expression of the fusion protein, we also generated transgenic lines expressing YFP-HR3 from the RPW8.2 promoter. As expected, expression of YFP-HR3 was mostly confined to haustorium-invaded epidermal cells and YFP signals were mostly or exclusively found in the EHM (Fig. 4D). To test if YFP-HR4 is also localized to the EHM, we inoculated YFP-HR4 expressing N. benthamiana leaves with Gc UCSC1, which is weakly infectious on N. benthamiana (Xiao et al., 2003). We also observed YFP signal as small punctae around the haustorium as well as in the cytoplasm (Fig. 4C). All T1 transgenic plants expressing these fusion proteins (> 20 for each construct) were as susceptible as Col-gl to Gc-UCSC1 (not shown), agreeing with the nonfunctional nature of these fusion proteins in defense as seen for YFP-RPW8.2 (Wang et al., 2010). To rule out the possibility that membrane proteins may be targeted by default to the EHM in the haustorium-invaded cells during the biogenesis of the EHM, we expressed the PM-localized membrane protein SYP131 (Uemura et al., 2004) tagged with YFP from the RPW8.2 promoter in Col-gl. YFP-SYP131 showed exclusive PM localization in haustorium-invaded cells (Fig. 5C), indicating that EHM-targeting of the YFP-tagged RPW8 homologs is attributable to the properties of these proteins rather than a consequence of activation of a default EHM-oriented trafficking pathway.

YFP-HR3 Is Localized to the Papilla

Intriguingly, we also noticed that YFP-HR3 showed focal accumulation around the fungal penetration site where a callosic papilla is formed (Fig. 5A). This localization pattern is reminiscent of PEN1/SYP121, a syntaxin involved in penetration (Collins et al., 2003; Assaad et al., 2004). To determine the precise localization of YFP-HR3 in relation to the papilla, we stained callose in the papilla by Sirofluor and found that YFP-HR3 was not precisely colocalized with callose, as it was more concentrated in the middle and the rim of the bull’s-eye-shaped callosic papilla (inset in Fig. 5A). Interestingly, we never observed signal from RPW8.2-YFP (RFP) or YFP (RFP)-RPW8.2 in the papilla (Fig. 5, A and D). These observations suggest that papilla-localization of YFP-HR3 is attributable to HR3 and may reflect a cellular function of HR3 that is distinct from RPW8.2.

Homologs of RPW8 exist in Brassica species (Xiao et al., 2004) but their biological functions have not been determined. To test if EHM- and papilla localization is indeed an ancestral feature of the RPW8 protein family, we made and expressed YFP fusion (both at the N- and C terminus) constructs for two RPW8 homologs from B. oleracea, BoHRa and BoHRb (Xiao et al., 2004) from the RPW8.2 promoter in Arabidopsis. We found that over 65% T1 lines (> 40) transgenic for BoHRa-YFP or BoHRb-YFP had greatly reduced plant stature with spontaneous cell death lesions and powdery mildew resistance (Fig. 6, A and B), suggesting that these Brassica homologs probably contribute to basal resistance against powdery mildew. Interestingly, unlike HR3-YFP, YFP signal from BoHRa-YFP, albeit weaker compared with RPW8.2-YFP, was detectable in powdery mildew-invaded cells as puncta mostly around the haustorium, with strong accumulation in the fungal penetration site (Fig. 6C). However, we never observed strong diffuse YFP signal from BoHRa-YFP at the EHM, highlighting a clear difference from RPW8.2-YFP. As expected, all the T1 lines (> 30)
transgenic for YFP-BoHRa or YFP-BoHRb were susceptible to Gc UCSC1 (not shown) and the fusion proteins were also found in the papilla (Fig. 6D) and the EHM (Fig. 6E). These observations further support that the EHM-targeting and papilla-location feature had likely evolved in RPW8 homologs before the separation of Brassica and Arabidopsis. Considering the gradual adaptation process of powdery mildew, it seems likely that during the Arabidopsis-powdery mildew coevolutionary conflict, RPW8.2 has gained EHM-focused localization while losing its papilla-localization.

The EHM Is Fixed at the Haustorial Neck and Physically Separable from the Plasma Membrane and the Papilla

Triple localization (i.e. the EHM, the PM, and the papilla) of YFP-HR3, regardless of its relevance to the physiological functions of HR3, may provide a unique tool to interrogate the origin and biogenesis of the EHM and how these membrane compartments might be physically connected with or separated from each other. To test if the EHM is separable from the PM and the papilla, we made transgenic plants coexpressing YFP-HR3 from the 35S promoter and RPW8.2-RFP from the RPW8.2 promoter. Constitutive expression of YFP-HR3 allows PM-labeling by YFP-HR3 before powdery mildew infection, and possible direct lateral diffusion or translocalization of YFP-HR3 from the PM to the EHM during haustorial biogenesis. Leaf sections inoculated with Gc UCSC1 were prepared at 3 dpi and subjected to plasmolysis (incubation with 0.5M NaCl). Interestingly, in some cells, the shrunken cytoplasm with the YFP-HR3-labeled PM was pulled off the cell wall but got stuck at the haustorial neck, wrapping around the haustorial complex with an EHM labeled by both YFP-HR3 and RPW8.2-RFP (Fig. 7A). In other cells, the shrunken cytoplasm with YFP-HR3-labeled PM was nearly or completely torn off the haustorial complex; however, YFP-HR3 localized at the papilla and the EHM remained unaffected (Fig. 7, B and C). These observations demonstrate that the PM is physically separable from the EHM and the papilla, and support that (1) there indeed exists a physical barrier (i.e. the neck-band) at the haustorial neck (Gil and Gay, 1977) that fixes and separates the PM from the EHM, and (2) the papilla is an extracellular membrane compartment that is compartmentalized from both the PM and the EHM. The EHM Is physically separable from the PM and the papilla. Gc UCSC-infected leaves expressing YFP-HR3 (from 35S) and RPW8.2-RFP (from the RPW8.2 promoter) were subjected to plasmolysis (0.5M NaCl). A, Representative confocal image taken at 10 min after treatment. Note that the cytoplasm with YFP-HR3-labeled PM was shrunk around the seemingly unaffected haustorium labeled by both YFP-HR3 and RPW8.2-RFP. B and C, Representative confocal images taken at 30 min after treatment showing that the cytoplasm with YFP-HR3-labeled PM was nearly (B) or completely (C) detached from the haustorial complex with an intact EHM labeled by YFP-HR3 and RPW8.2-RFP. Note that YFP-HR3 localized to the papilla stained by PI (arrows in C) was also unaffected. Bar, 10 μm in A to C. C, Cytoplasm; H, haustorium.
likely formed via exosome secretion (Meyer et al., 2009). It is also important to note that despite that the cytoplasm in most affected cells was shrunk >5 times during plasmolysis, the size and shape of most haustorial complexes did not seem to have significant changes (Fig. 7), suggesting that the EHM and the fungal cell wall in particular may be rigid and resistant to high osmotic pressure.

YFP-HR3 Is Targeted to the Papilla and the EHM via Distinct Trafficking Pathways

To determine if YFP-HR3 is targeted to the papilla and the EHM via the same or distinct intracellular trafficking pathways, we first tested if there is lateral diffusion and/or an intracellular trafficking pathway between the PM and the EHM. FM4-64 is a lipophilic dye that labels the PM and tracks PM-derived endosomes to various subcellular compartments in a time-dependent manner in approximately 30 min (Bolte et al., 2004). While FM4-64 (20 μM in 0.002% Silwet L-77 aqueous solution) nicely labeled the PM in 5 min to 10 min after application of the dye and then was found to be colocalized with YFP-HR3 in the papilla in approximately 20 min, FM4-64 did not label the EHM in 45 min when all endosomal compartments should be labeled (Fig. 8A). However, if vacuum-infiltrated into the leaf tissue, FM4-64 could immediately stain the EHM (Fig. 8B). Given that the haustorial membrane lobes and the haustorial neck in particular were more strongly stained by FM4-64 (Fig. 8B), we speculate that the haustorial neckband as the presumable diffusion barrier is probably disrupted by vacuum infiltration, resulting in diffusion of the FM4-64 into the extrahaustorial matrix from the apoplast. This observation suggests that while membrane lipids or proteins from the PM can reach the papilla likely via an endosomal trafficking pathway, there is no or little (undetectable) lateral diffusion or endosomal trafficking from the PM to the EHM.

We then determined if YFP-HR3’s papilla- and the EHM-localization can be inhibited by the same pharmacological inhibitor. It has been shown that Brefeldin A (BFA), a fungal toxin widely used to block multiple intracellular vesicle trafficking pathways, can inhibit focal accumulation of both GFP-PEN1 and callose deposition to the papilla (Nielsen et al., 2012). We treated leaves of plants transgenic for 35S::YFP-HR3 and R82p::RPW8.2-RFP with BFA (300 μM) and found that while EHM localization of YFP-HR3 and RPW8.2-RFP appeared unaffected, both focal accumulation of YFP-HR3 and callose to the papilla were similarly diminished (Fig. 8, C and D). These observations indicate that EHM-oriented protein targeting and papilla-focused protein accumulation are realized via two distinct trafficking pathways.

Figure 8. Papilla-accumulation of YFP-HR3 is BFA sensitive while its targeting to the EHM is not. A, Surface-applied FM4-64 (20 μM) in 0.002% Silwet L-77 stained the PM and the papilla in 20 min (image on the left) but did not stain the EHM labeled by YFP-HR3 in 45 min (image on the right). B, When vacuum-infiltrated into leaf tissue (see “Materials and Methods”), FM4-64 could stain the EHM (arrow) labeled by RPW8.2-YFP as well as the haustorial membrane lobes. Notably, the haustorial neck is often more strongly stained (arrowhead). C, BFA inhibits focal accumulation of YFP-HR3 and callose to the papilla. Quantification of relative fluorescence intensity for YFP-HR3 and Sirofluor-stained callose at the papilla in confocal images. Data represent means ± se (n = 6) from one of two independent experiments. Student’s t test was used to compare the differences between treatments (* indicates P < 0.01, ** indicates P < 0.001). D, Representative images showing localization of YFP-HR3 to the PM, the papilla, and the EHM at 45 hpi in epidermal cells infiltrated with 1% DMSO buffer (upper panel) or 300 μM BFA (lower panel) at approximately 6 hpi from the base of the leaf. The EHM was labeled by RPW8.2-RFP and the callose was stained with Sirofluor. Note that both YFP-HR3 and callose were diminished in the papilla due to BFA treatment, but YFP-HR3 and RPW8.2-RFP at the EHM were unaffected. Bar, 10 μm in A to E. H, Haustorium; p, papilla.
DISCUSSION

In this study, we showed that HR3, the probable progenitor of the RPW8 family, HR2, and possibly HR1 play a positive role in basal resistance and that EHM-localization is probably a conserved feature of the RPW8 family. Using HR3's localization to the PM and papillae, we also collected evidence to suggest that the EHM is unlikely formed via PM invagination and the PM-to-EHM endocytic trafficking makes little or no contribution to the EHM biogenesis.

Our previous studies showed that RPW8.1 and RPW8.2 evolved recently in Arabidopsis after its separation from Brassica species, while RPW8 homologs more closely related to HR3 are found in B. oleracea and B. rapa and appear to be under purifying selection (Xiao et al., 2004; Orgil et al., 2007). Though anticipated, whether these RPW8 homologs are involved in basal resistance against powdery mildew remained undetermined. Through genetic and localization analyses we now have demonstrated that HR1, HR2, and HR3 contribute to basal resistance to powdery mildew and that all the Arabidopsis RPW8 family proteins including RPW8.1 (Ma et al., 2014) are probable EHM-residents. HR1, HR2, and HR3 probably have a partially redundant function in basal resistance but this collective role cannot be accurately measured without a triple knockout mutant. Because these three genes are tandemly arrayed in an 8 Kb region, making double or triple mutants by genetic crossing is extremely difficult. We tried to silence HR1 and HR2 in the HR3-kd1 background but did not see any significant additional disease susceptibility (data not shown). Targeted mutagenesis using gene editing tools such CRISPR/Cas9 will be used to generate a triple mutant to assess the overall role of these three genes in the future. HR4 is believed to be the newest member of the RPW8 gene family, likely created from the deletion of RPW8.1 and RPW8.2 that resulted from a transposition event (Xiao et al., 2004). Hence, it is not a surprise to us that HR4 did not appear to contribute to basal resistance against powdery mildew and YFP-HR4 was not detectable in Arabidopsis. Interestingly, HR4 has been shown to be induced by powdery mildew (Chandran et al., 2011) and the beneficial fungus Trichoderma (Sáenz-Mata and Jiménez-Bremont, 2012), suggesting that HR4 is involved in biotic stress response. Unfortunately, no T-DNA lines for HR4 are available. Future targeted mutagenesis is required for understanding the biological function of HR4.

Taken together, it seems that RPW8.2’s capability in activating haustorium-targeted defense is not entirely new, but instead a capability that stemmed from and then surpassed that of the older family members. A critical question then is how RPW8.2-mediated resistance is mechanistically differentiated from the original function of the HR3-like progenitor (Xiao et al., 2004). Our present studies provided some insights into this question. First, RPW8.2-YFP can accumulates at high levels in the EHM, whereas HR3-YFP is undetectable, yet triggers massive cell death (Figs. 2, 3, and 6). This suggests that the C-tail of HR3 may serve as an auto-inhibitory domain that needs to be intact for proper function of HR3, whereas this constraint is probably relaxed if not completely removed for RPW8.2. Second, because the chimeric protein R82n-HR3c-YFP but not HR3n-R82c-YFP behaved similarly as RPW8.2-YFP in defense and EHM-targeting (Fig. 3), we can conclude that sequence diversification in the N-terminal 147 amino acids between HR3 and RPW8 largely underscores their functional differences. A detailed mutational analysis is needed to identify key residues for improved defense activity in RPW8.2 or higher activity in triggering cell death in HR3 (when its C terminus is fused with YFP) from a total of 42 amino acid substitutions between these two proteins in this region (Fig. 3A). Third, YFP-HR3 but not YFP-RPW8.2 was found in the papilla, suggesting that HR3 might play a role in penetration resistance and a signal in HR3 for papilla localization might have been lost in RPW8.2, which seems to be reasonable as RPW8.2 apparently triggers haustorium-targeted defense at the postpenetration stage. It should be pointed out however that functional enhancement of RPW8.2 from an HR3-like gene may be partially attributable to the RPW8.2 promoter, which renders powdery mildew inducible, epidermal cell-specific expression of RPW8.2 (Wang et al., 2009) and showed higher activity when driving HR3-YFP expression as reflected by a higher proportion of T1 lines with lethal cell death (Fig. 2D). Thus, functional evolution of RPW8.2 from an HR3-like progenitor gene probably necessitated DNA sequence diversification in the 5’ regulatory region for enhanced, powdery mildew-inducible expression, and in the coding region for higher efficiency in defense activation while conserving the amino acids for EHM-targeting.

The origin and biogenesis of the EHM is of great interest to researchers in the field because of its apparent importance in understanding the host-pathogen interaction but unfortunately remains largely undetermined. The absence of eight PM-localized proteins in the EHM (Koh et al., 2005) and the presence of RPW8.2 exclusively in the EHM (Wang et al., 2009) suggest that the EHM and the PM are more likely two distinct membranes with different origins. However, theoretically, one cannot exclude the possibility that the EHM is initially derived from invagination of the PM when the haustorium develops from the tip of the fungal penetration peg inside the host epidermal cell, and then differentiates into a membrane distinct from the PM by selective exclusion of certain PM proteins and specific recruitment of other proteins such as RPW8.2. One of the difficulties we face when addressing this question is the lack of a good protein marker that is localized to the PM and the EHM at high levels. In this regard, we were lucky to find that YFP-HR3 is localized in the PM, the papilla, and the EHM (Fig. 5), because this localization feature could be very valuable for testing the above possibility and assessing possible interconnections between the three membrane compartments. Based
on our results from YFP-HR3 localization and FM4-64 staining analyses, we suggest that the EHM is unlikely derived from PM invagination; rather, it is more likely synthesized de novo via exocytosis with possible contribution from other intracellular membrane compartments. Specifically, our above reasoning is supported by the following three aspects. First, we performed plasmolysis of haustorium-invaded epidermal cells expressing YFP-HR3 and RPW8.2-RFP and found that YFP-HR3 signal was virtually intact in the EHM throughout the plasmolysis and was separable from the shrunk cytoplasm with YFP-HR3-labeled PM (Fig. 7). This demonstrates that the EHM is indeed a membrane compartment distinct and physically separable from the PM, which allows isolation of individual haustorial compartments containing the EHM (Fig. 5B). Second, we found that PM-incorporated FM4-64 failed to reach the EHM in 45 min although it could reach the papilla in 20 min under conditions when the integrity of the haustorial complex is not affected (Fig. 8A). This indicates that there is a diffusion barrier (most likely attributable to the haustorial neckband) between the PM and the EHM, and implies that endocytic trafficking from the PM to the EHM either does not occur at all or is minimal. Indeed, when such a diffusion barrier is likely disrupted upon vacuum infiltration of a leaf section containing haustorial complexes in the presence of 0.002% surfactant Silwet L-77, FM4-64 can readily and immediately stain the EHM and the haustorial membrane (Fig. 8B). In line with this inference, an exocytosis pathway defined by a SNARE complex defined by Vamp721/Vamp722 and SNAP33 has recently been shown to be required for efficient targeting of RPW8.2 vesicles to the EHM (Kim et al., 2014). Third, consistent with the above results, we also found that YFP-HR3’s localization to the papilla is BFA sensitive, whereas EHM-targeting of YFP-HR3 (and RPW8.2-RFP) is BFA insensitive (Fig. 8, D and E). Papilla-localization of YFP-HR3 is reminiscent of an earlier report that YFP-PEN1 and callose are delivered to the papilla via a BFA-insensitive pathway, which requires the ADP ribosylation factor-GTP exchange factor GNOM (Nielsen et al., 2012).

Thus, it is likely that the same GNOM-dependent pathway is engaged for delivery of YFP-HR3 to the fungal penetration sites. Given that, unlike in animals, BFA mainly inhibits certain endosome recycling pathways and there exists BFA-insensitive exocytic trafficking in plants (Richter et al., 2005). Unless otherwise indicated, seeds were sown in Sunshine Mix no. 1 or Metro-Mix 360 soil (MD Plant & Suppliers) and cold-treated (4°C for 1 to 2 d). Seedlings were kept under 22°C, 75% RH, short-day (8 h light at approximately 125 μmol·m⁻²·s⁻¹, 16 h dark) conditions for 5 to 6 weeks before pathogen inoculation and/or other treatments.

Pathogens Strains, Inoculation, and Phenotyping

Powdery mildew isolate Golovinomyces cichoracearum UCSC1 (Gc-UCSC1) was maintained on live Col-0 or pad4-1 mutant plants for generation of fresh conidia for inoculation purposes. Inoculation, visual scoring, photographing, and quantification of disease susceptibility were done as described in Materials and Methods.
DNA Constructs and Generation of Transgenic Lines

All primer sequences and related information are summarized in Supplemental-Table 1. Overexpression constructs for HR1, HR2, HR3, and HR4 were created after amplification of the genomic sequence including approximately 1000 bp of the 5′ UTR/promoter region (496 bp in the case of AHHR3) from the T2I2E2 BAC clone obtained from the Arabidopsis Biological Resource Center into the binary vector pkmBi downstream of the 3SS promoter (Mylene and Botella, 1998).

The pCX-DG TA cloning vector described in Chen et al. (2009) was modified to include an approximately 1.8 kb RFPW82 promoter region by releasing the 35S:GFP fragment upstream of the XcmI and plant NOS terminator using EcoRI digestion and replacement (pCX-DG-R2sp). We used this modified TA cloning vector to express the full-length HR3 and HR3 variants, and HR3-RFPW82 chimeric genes.

HR1, HR2, HR3, and HR4 genes were also translationally fused to YFP at the N terminus under control of the 5′SS promoter using the pEarleyGate Gateway compatible vector series (Earley et al., 2006). Recombination reactions were completed using pENTR/D-TOPO clones of the full genomic DNA sequences. Gateway LR Clonase II enzyme mix was used to complete the recombination reaction between the entry clones and destination vector (pEGC104).

Chimeric domain swapping constructs between the N-domain (amino acid 1 to 147; HR3n) and the C-domain (amino acid 148 to 213; HR3c) were created using nucleotides designed at the ends of RFPW82 and the C-domain (amino acid 146 to 174; R62c) of RFPW82 were created using Ncol adapted primers designed at the ends of R62c. The 5′SS predicted coiled-coil domain of each gene, HR3n-RRS3-R82c and R82c-R82n-HR3c chimeric genes were translationally fused to YFP at the C terminus under control of either the HR3 or RFPW82 promoter using the previously mentioned pZZPYFP22′ homedake vector (Wang et al., 2007). Additionally, HR3n-R82c-YFP was also cloned into the homedake pCX-DG-R2sp.

TA cloning using the modified binary vector pCX-DG-R2sp was also used for a variety of other constructs including: YFP-HR1, YFP-HR2, YFP-HR3, YFP-HR4 and RFPW82-RFP. All generated constructs in binary vectors were introduced into Agrobacterium strain GV3101 and stable transgenic Arabidopsis plants were generated by floral dip (Clough et al., 2000).

Pharmacological Treatments and Gel Blotting

Fully expanded leaves of approximately 7 week-old RFW8.2-YFP or YFP-HR3/RFPW82-RFP Col-0 background plants were detached from the base of the plant and inserted into sterilized Murashige and Skoog agar medium in petri dishes. Detached leaves were inoculated evenly with Gc-UCSC1 and at different time points (0, 12, or 16 hpi) leaf sections (approximately 0.25 cm²) were examined using a model no. LSM710 confocal microscope (Zeiss) as previously described in Wang et al. (2009, 2013). Image data were processed using Zen software.

Acknowledgments

We thank Frank Coker for maintaining the growth facility, Dr. Ying Wu for critical reading of this manuscript, and two anonymous reviewers for their constructive comments, which helped us to improve the manuscript.

Received October 5, 2016; accepted November 15, 2016; published November 17, 2016.

Literature Cited


Controlling the MAPK pathway is required for the timely assembly of papillae. Mol Biol Cell 15: 5118–5129

For western blotting, Arabidopsis leaves infected with Gc-UCSC1 were collected at 5 dpi for preparation of total microsomal proteins as described in Wang et al. (2007). An anti-GFP antibody (A290; Abcam) was used to detect YFP-fusion proteins from various Arabidopsis transgenic lines.

Isolation of Haustorial Complexes

This protocol is modified from the “Isolation of intracellular hyphae by isopycnic centrifugation technique” described in Pain et al. (1994). Extracted haustoria suspensions were obtained from pooled T2 lines of appropriate transgenic backgrounds between 7 and 8 dpi with Gc-UCSC1. Approximately 1 to 2 total grams of infected leaf tissue was macerated 2× in a conventional kitchen blender in a minimum volume of 20 to 30 mL of fresh, chilled haustoria extraction buffer (3-[N-morpholino]-propane sulfonic acid 0.02 M, pH 7.2 containing 0.2 M Suc) for 45 to 60 s. After each 1× blending procedure, macerated plant material was filtered through a 40 μm nylon mesh material using vacuum filtration to remove large fragments of plant debris as most mature Gc UCSC1 haustoria are between 15 and 25 μm in size. Final filtrates were centrifuged at 1080g for 15 min at 4°C. Pelleted material containing haustoria was resuspended in 2 to 3 mL of haustoria extraction buffer and kept at 4°C before imaging or further analysis.

Confocal Microscopy

Laser scanning confocal microscopy was done using a model no. LSM710 confocal microscope (Zeiss) as previously described in Wang et al. (2009, 2013). All images and videos presented are single optical sections or Z-stack projected of 15 to 30 images unless otherwise indicated. For FM-64 staining, detached leaf sections (approximately 0.25 cm²) were submerged in 20 μM FM-64 (Molecular Probes) in water for 15 to 45 min. For PI staining, detached leaf sections (approximately 0.25 cm²) were submerged in 0.5% PI solution for 45 to 60 min then washed briefly (10 to 15 min) in water before imaging. Sirofluor staining of leaf sections for callose was conducted with a 0.1 mg mL⁻¹ dissolved in a 1% DMSO solution (Biologicals). Image data were processed using Zen 2009 Light Edition and Adobe Photoshop CS5.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis genome initiative or GenBank/EMBL database under accession numbers AT3G50450 (HR1), AT3G50460 (HR2), AT3G50470 (HR3), AT3G50480 (HR4), AA225587 (BoHRa), and AA225588 (BoHRb).
and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science 310: 1180–1183


8

Copyright © 2017 American Society of Plant Biologists. All rights reserved.


612


