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Dominant negative RPW8.2 fusion proteins reveal the importance of haustorium-oriented protein trafficking for resistance against powdery mildew in Arabidopsis

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Abbreviations: EHM, extrahaustorial membrane; PM, plasma membrane; eds, enhanced disease susceptibility.

Powdery mildew fungi form feeding structures called haustoria inside epidermal cells of host plants to extract photosynthates for their epiphytic growth and reproduction. The haustorium is encased by an interfacial membrane termed the extrahaustorial membrane (EHM). The atypical resistance protein RPW8.2 from Arabidopsis is specifically targeted to the EHM where RPW8.2 activates haustorium-targeted (thus broad-spectrum) resistance against powdery mildew fungi. EHM-specific localization of RPW8.2 suggests the existence of an EHM-oriented protein/membrane trafficking pathway during EHM biogenesis. However, the importance of this specific trafficking pathway for host defense has not been evaluated via a genetic approach without affecting other trafficking pathways. Here, we report that expression of EHM-oriented, nonfunctional RPW8.2 chimeric proteins exerts dominant negative effect over functional RPW8.2 and potentially over other EHM-localized defense proteins, thereby compromising both RPW8.2-mediated and basal resistance to powdery mildew. Thus, our results highlight the importance of the EHM-oriented protein/membrane trafficking pathway for host resistance against haustorium-forming pathogens such as powdery mildew fungi.

Introduction

Powdery mildew is one of the most common and important plant diseases caused by ascomycete fungi belonging to the order of *Erysiphales*.[1,2] Several distinct powdery mildew resistance mechanisms have been characterized in plants. For example, plants use intracellular immune receptors traditionally named resistance (R) proteins to detect the presence or virulence activity of a specific effector from powdery mildew and subsequently activate resistance to a strain of powdery mildew expressing the recognized effector.[3-6] In contrast, the disease resistance (*R*) gene locus *RPW8* confers broad-spectrum resistance in *Arabidopsis thaliana* to powdery mildew fungi.[7] This locus contains 2 homologous atypical *R* genes, *RPW8.1* and *RPW8.2* (collectively referred to as *RPW8* unless otherwise indicated). Protein expression and localization studies revealed that (i) both proteins are induced by powdery mildew infection, and (ii) RPW8.1 is only expressed in mesophyll cells while RPW8.2 is mainly expressed in the mildew-infected epidermal cells albeit detectable in the mesophyll cells underneath the infection site.[8,9] Our recent work showed that these 2 proteins seem to spatially collaborate to activate defense responses.[10] Most interestingly, RPW8.2 is specifically targeted to the extra-haustorial membrane (EHM), which is believed to be a host cell-derived interfacial membrane encasing the fungal haustorium.[9,11,12] The EHM-specific localization of RPW8.2 correlates with accumulation of hydrogen peroxide (H_2O_2) around the EHM and formation of a callose-enriched encasement of the haustorial complex, therefore providing a cell biological basis for RPW8-mediated broad-spectrum resistance.[9,13] More recently, we demonstrated that RPW8.2 (174 amino acids in total) possesses 2 basic residue–enriched EHM-targeting motifs, which together with the putative N-terminal

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signal peptide or transmembrane domain, constitute a minimum 60 amino acid sequence that is necessary and sufficient to target YFP to the EHM.[14] Because protein trafficking to other subcellular compartments such as the plasma membrane (PM) appears to be normal in the haustorium-invaded cells,[12,14] RPW8.2's specific localization to the EHM suggests the existence of an EHM-oriented specific protein/membrane trafficking pathway. Our earlier work also showed that although defense activated by RPW8.2 requires the conserved salicylic acid (SA)-dependent signaling pathway,[15,16] EHM-targeting of RPW8.2 to the EHM is SA-independent.[9] For example, RPW8.2-YFP expressed in SA-signaling defective *eds1-2* or *pad4-1* mutant is still targeted to the EHM.[9] Conversely, when EHM-oriented trafficking is disrupted by application of a cytoskeleton inhibitor cytochalasin E or via overexpression of an actin depolymerizing factor (ADF6), RPW8.2 accumulates as punctate vesicles in the infected epidermal cells and these vesicles appear to correlate with cell death activation.[9] These observations suggest that defense function of RPW8.2 may not be contingent to its EHM-localization. However, since pharmacological or genetic inhibition of the actin cytoskeleton probably blocks multiple trafficking pathways, the cell death observed in affected plants expressing RPW8.2-YFP may not be (solely) attributable to accumulation of RPW8.2 vesicles along the EHM-oriented trafficking pathway. Thus, the role of the EHM-oriented trafficking in RPW8.2-mediated defense has yet to be unequivocally defined. Here, we show that several YFP-tagged fusion proteins in which a small PM protein Lti6b[17] is fused with N-terminally truncated RPW8.2 (i.e., YFP-Lti6b-RPW8.2ΔNt; Y6R82ΔNt for short) exert a dominant negative effect over the RPW8.2 wild-type protein. That is: expression of those fusion proteins that show EHM-docking not only compromises EHM-targeting of RPW8.2-YFP but also largely abrogates RPW8.2-mediated cell death and resistance. In addition, expressing such Y6R82ΔNt proteins in plants lacking *RPW8* results in enhanced disease susceptibility compared with susceptible wild-type plants, implying the existence of other EHM-localized defense proteins whose trafficking is also affected. These observations highlight the importance of EHM-oriented protein trafficking for host resistance against haustorium-forming pathogens such as powdery mildew fungi.

Results

YFP-tagged LTI6b-RPW8 chimeric proteins show EHM-oriented docking

In the course of identifying EHM-targeting motifs in RPW8.2, we initially adopted a strategy deployed by Le Maout and colleagues to identify the targeting motif required for basolateral membrane localization of the inwardly rectifying potassium channel Kir 2.3 in certain renal epithelial cells.[18] That strategy involves fusion of the candidate C-terminal tail of Kir2.3 with CD4, a cell-surface protein without apparent polarity, followed by serial truncations to identify minimal size of the Kir2.3's C-tail that renders basolateral membrane localization of CD4.[18] We chose Lti6b (At3g05890), a small (55 amino acids in total) PM-localized protein as a membrane cargo.[17] Lti6b was translationally fused with RPW8.2 that lacks its N-terminal transmembrane domain or signal peptide (amino acid 1 to 22), and YFP was added to the N-terminus of LTI6b to make a YFP-LTI6b-RPW8.2ΔNt22 fusion construct (designated Y6R82ΔNt22). The DNA construct was placed under control of the *RPW8.2* promoter and stably expressed in Arabidopsis accession Col-0 lacking *RPW8*.[7] Confocal imaging showed that expression of Y6R82ΔNt22 was nicely induced by infection from powdery mildew isolate *Golovinomyces cichoracearum* (*Gc*) UCSC1. In ~75% invaded epidermal cells, the fusion protein was detected in varied-sized puncta randomly scattered in invaded cells (**Fig. 1A**). In ~25% invaded cells however, the fusion protein was detected as puncta or small patches around the EHM (**Fig. 1B**), suggesting that the vesicles carrying this fusion protein partially retains the ability to dock to the EHM. However, since limited diffuse YFP signal was detected in the EHM, it appears that the chimeric protein was largely unable to fuse with the EHM. Consistent with this observation, all 36 Col-0 T1 independent transgenic lines expressing this construct were fully susceptible to powdery mildew (not shown), suggesting that this chimeric protein is not functional in defense activation. Nevertheless, we speculated that the assumed EHM-targeting motif(s) in RPW8.2 is (partially) functional in Y6R82ΔNt22 to enable docking of this fusion protein to the EHM. We thus made 11 additional chimeric constructs in which different sized N-terminally truncated *RPW8.2* variants were each translationally fused with YFP-Lti6b (**Table 1**), hoping to identify amino acid regions of RPW8.2 required for EHM-oriented targeting (docking).

We generated at least 24 T1 transgenic lines for each construct and examined them for protein localization. Interestingly, construct #2 (*Y6R82ΔNt31*) to #7 (*Y6R82ΔNt101*) all produced fusion proteins that were mostly found in random puncta, with only occasional or rare (i.e., in ~10% or fewer invaded cells) EHM-association as seen for Y6R82ΔNt22 (**Table 1**). Surprisingly, Y6R82ΔNt102 (which contains a shorter RPW8.2 C-tail) exhibited the most frequent (~65%) EHM-association (**Fig. 1C**) with more apparent EHM-docking (inset in **Fig. 1C**). However, the fusion proteins from the remaining constructs only exhibited randomly distributed puncta unrelated to the EHM (see one example in **Fig. 1D**). We thus used transgenic line expressing Y6R82ΔNt102 for more detailed characterization of its EHM-docking. The earliest EHM-oriented vesicle aggregation of Y6R82ΔNt102 was detectable at ~16 hrs post-inoculation (hpi) and more obvious at 20 hpi (**Fig. 1E**), which roughly concurs with the earliest detection of RPW8.2-YFP as diffuse signal in the EHM.[9] Vesicles were seen rapidly moving to the periphery of the haustorium via EHM-oriented strands (which may represent actin cables) (**Fig. 1F**; Supplemental movie 1). These vesicles (0.5-1.0 μm) were 2–5 times larger than those of RPW8.2-YFP (0.1-0.5 μm) that are rarely seen presumably because their trafficking is much quicker.[9]

Our above observations seemed to suggest that amino acids 103 to 174 in RPW8.2 confers EHM-docking and that amino acids 103 to115 are most critical for this trafficking property.

These results were different or even contradictory to what we later found using the functional (both in terms of defense and trafficking) *RPW8.2-YFP* fusion gene as template for a large-scale mutational analysis through which we identified amino acids 20–30 and 95–100 to be the core EHM-targeting signals in RPW8.2.[14] It is possible that the C-terminal portion (especially from amino acids103 to 115) of RPW8.2 may contain a cryptic EHM-targeting signal which when combined with the transmembrane domains from Lti6b may enable EHM-oriented trafficking. Alternatively, combining the RPW8.2 C-terminal 71 amino acids with YFP-Lti6b may generate a chimeric protein that somehow can more readily enter the EHM-oriented trafficking pathway.

None of the Col-0 T1 transgenic lines (>24 examined for each construct) expressing any of the YFP-Lti6b–tagged RPW8.2 variants showed resistance to powdery mildew. It is possible that the addition of Lti6b and/or the N-terminal tagging of YFP-Lti6b to RPW8.2 may change the conformation and/or topology of RPW8.2, resulting in nonfunctional fusion proteins dissimilar to the endogenous RPW8.2 protein or RPW8.2-YFP. We thus abandoned this approach for searching for physiologically relevant EHM-targeting signals.

Expression of YFP-Lti6B-RPW8 fusion proteins compromises basal resistance to powdery mildew

Unexpectedly, we observed by the naked eye that 20–35% of the T1 lines (>24 for each construct) expressing those YFP-Lti6b–tagged RPW8.2 variants with EHM-docking showed enhanced disease susceptibility ('eds') to powdery mildew isolate *Gc* UCSC1 (one example in **Fig. 2A**). However, no obvious 'eds' phenotype was observed in T1 lines expressing fusion proteins that lacked EHM-docking or expressing YFP-Lti6b alone (one example in **Fig. 2B**). We speculated that those YFP-Lti6b-R82 proteins capable of entering the EHM-oriented trafficking pathway might be able to produce a dominant negative effect over endogenous EHM-resident proteins, hence the 'eds'. To

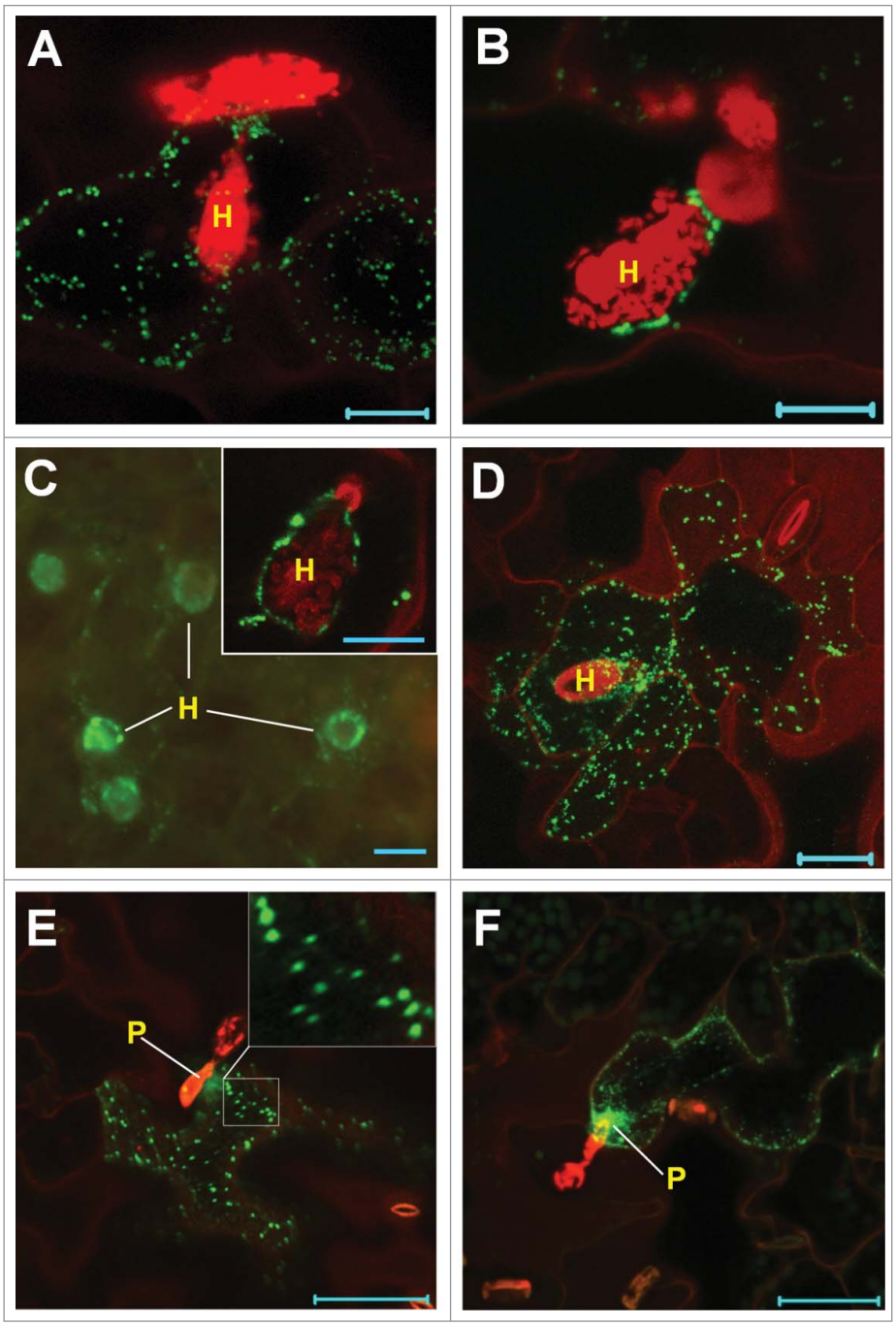

Figure 1. Localization of YFP-Lti6b–tagged RPW8.2 variants in haustorium-invaded cells. Six week-old transgenic plants were inoculated with *Gc* UCSC1 and infected leaves were subjected to confocal imaging. H, haustorium; P, penetration site. Bar represents 10 μm in A-D, 50 μm in E and F. (**A**) A representative confocal images (Z-stack) showing random localization of Y6R82ΔNt22 vesicles in ~75% haustorium-invaded cells. (**B**) A representative confocal image (single optical section) showing EHM-docking of Y6R82ΔNt22 puncta in ~25% haustorium-invaded cells. (**C**) A representative epifluorescent image showing EHM-oriented localization of Y6R82ΔNt102. Inset is a confocal image of one haustorium-invaded cell. (**D**) A representative confocal image (Z-stack) showing random localization of Y6R82ΔNt114 vesicles in all haustorium-invaded cells. (**E**) A representative confocal image (Z-stack) showing earliest (16-20 hpi) EHM-oriented vesicle docking of Y6R82ΔNt102. Note the large-sized vesicles (0.5–1.0 μm) in the inset. (**F**) A representative confocal image (single optical section) showing EHM-oriented vesicle strands of Y6R82ΔNt102 (see live targeting in Movie 1 in the online supplemental information).

Table 1. RPW8.2 DNA constructs and protein localization patterns

Name of constructs	Nature of mutations	Localization pattern*
YFP-Lti6b (Y6)	Control	Plasma membrane
R82-RFP	Control	EHM
Y6R82ΔNt22	Δ1-22 amino acids	EHM (puncta); ++
Y6R82ΔNt31	Δ1-31 amino acids	EHM (puncta); +
Y6R82ΔNt42	Δ1-42 amino acids	EHM (puncta); +
Y6R82ΔNt93	Δ1-93 amino acids	EHM (puncta); +
Y6R82ΔNt97	Δ1-97 amino acids	EHM (puncta); +
Y6R82ΔNt99	Δ1-99 amino acids	EHM (punctate); +
Y6R82ΔNt101	Δ1-101 amino acids	EHM (puncta); +
Y6R82ΔNt102	Δ1-102 amino acids	EHM (puncta); +++
Y6R82ΔNt115	Δ1-115 amino acids	Random puncta only
Y6R82ΔNt118	Δ1-118 amino acids	Random puncta only
Y6R82ΔNt120	Δ1-120 amino acids	Random puncta only
Y6R82ΔNt123	Δ1-123 amino acids	Random puncta only
$R82^{N26+N95}$-YFP	KDRSVT26-31NAAIRS + RKKFRY95-100NAAIRS	Random puncta only

*Localization patterns of fusion proteins expressed from the *RPW8.2* promoter in haustorial invaded cells. At least 24 T1 lines were examined. +, <10% cells with EHM-docking; ++, 10–25% cells with EHM-docking; +++, >25% cell with EHM-docking.

test this idea, we did a comparative phenotypic analysis with Col-0 lines expressing Y6R82ΔNt42, Y6R82ΔNt102, $R82^{N26+N95}$-YFP, or YFP-Lti6b, along with Col-0 wild-type and Col-*nahG*, a transgenic line known to have 'eds' to powdery mildew due to SA-deficiency.[16] The $R82^{N26+N95}$-YFP is a DNA fusion construct in which amino acids 26–31 and 95–100 falling into the 2 EHM-targeting motifs of RPW8.2 were each replaced by the 6 amino acids "NARRIS" such that the fusion protein loses its EHM-targeting signal yet with minimal structural perturbation when compared to the functional RPW8.2-YFP protein.[14] Compared with Col-0, plants of selected representative homozygous T3 transgenic lines expressing Y6R82ΔNt42 or Y6R82ΔNt102 showed clear 'eds' (though not as strong as that of Col-*nahG*), whereas plants expressing YFP-Lti6b or $R82^{N26+N95}$-YFP showed no or only marginally increased susceptibility (**Fig. 2C-2D**). These results suggest that Y6R82ΔNt42 and Y6R82ΔNt102 in particular may be more potent in interfering the normal EHM-oriented trafficking than $R82^{N26+N95}$-YFP. Because Col-0 does not contain *RPW8.1* and *RPW8.2*[7] but contains 4 homologs of *RPW8* (*HR1, HR2, HR3* and *HR4*), it is possible that these RPW8 homologs may be EHM-resident proteins and contribute to basal resistance in Col-0. Hence, expression of Y6R82ΔNt42 and Y6R82ΔNt102 might negatively affect EHM-targeting and function of these proteins.

Expression of YFP-Lti6B-RPW8 fusion proteins compromises RPW8-mediated resistance to powdery mildew

An extrapolation of the above results is that expression of Y6R82ΔNt42 and Y6R82ΔNt102 may exert a dominant negative effect over RPW8.2′s resistance function. To test this, we individually introduced Y6R82ΔNt42, Y6R82ΔNt102 and $R82^{N26+N95}$-YFP into S5, which is a Col-0 line transgenic for a single copy of *RPW8.1* and *RPW8.2*.[15] We examined the disease phenotypes of S5 plants and S5 transgenic T1 lines. As expected, we found that more than half of S5 T1 lines transgenic for *Y6R82ΔNt42* or *Y6R82ΔNt102* were susceptible or moderately susceptible to powdery mildew. In contrast, only 3 of the 24 S5 T1 lines transgenic for *$R82^{N26+N95}$-YFP* were slightly more susceptible, the remaining lines were similar to S5 plants (**Fig. 3A**). The disease phenotypes were confirmed with at least 2 independent homozygous T3 lines for each construct based on quantitative assays (**Fig. 3B**). Based on these data, it is obvious that RPW8-mediated resistance was largely abolished due to ectopic expression of Y6R82ΔNt42 or Y6R82ΔNt102 in particular, whereas ectopic expression of $R82^{N26+N95}$-YFP had little effect.

Expression of YFP-Lti6b-RPW8.2 fusion proteins reduces EHM-targeting of RPW8.2

One likely mechanistic explanation for the dominant negative effect of Y6R82ΔNt42 or Y6R82ΔNt102 over RPW8.2 is that these fusion proteins could enter the EHM-oriented trafficking pathway and sequester relevant trafficking components engaged for RPW8.2′s EHM-targeting, thereby resulting in (partial) loss of RPW8.2′s EHM-localization and defense function. To obtain additional evidence for this speculation, we generated Col-0 transgenic lines co-expressing Y6R82ΔNt42 or Y6R82ΔNt102 and RPW8.2-RFP (R82-RFP) through agrobacterium-mediated stable transformation. While 30% (20 of 66) T1 transgenic lines expressing R82-RFP exhibited resistance to powdery mildew, none of the 6 T1 lines co-expressing Y6R82ΔNt42 with R82-RFP and the 10 T1 lines co-expressing Y6R82ΔNt102 with R82-RFP showed similar level of resistance, further validating the results from S5 transgenic lines (**Fig. 3**). Confocal imaging of leaves inoculated with *Gc* UCSC1 showed that Y6R82ΔNt102 was docked around the EHM labeled by R82-RFP but there was no diffuse YFP signal in the EHM (**Fig. 4A**). When more Y6R82ΔNt102 puncta was detected in the haustorium-invaded cells, R82-RFP signal was in most cases very weak or undetectable (**Fig. 4B**), suggesting likely interference of EHM-oriented trafficking of R82-RFP by Y6R82ΔNt102. By counting the number of R82-RFP–labeled (EHM) haustoria versus the total number of haustoria (stained by propidium iodide) in *Gc*

UCSC1-inoculated leaves of 3 representative T1 lines for each construct, we found that EHM-targeting efficiency of R82-RFP was reduced from 85.8% to 35.5% due to co-expression of Y6R82ΔNt42 and to 24.1% due to co-expression of Y6R82ΔNt102. In contrast, co-expression of $R82^{N26+N95}$-YFP caused only mild reduction (from 85% to 67.4%) in RPW8.2-RFP's EHM-localization (**Fig. 4C**).

Combined, these results indicate that disruption of EHM-localization of RPW8.2 by dominant negative Y6R82ΔNt42 or Y6R82ΔNt102 in particular inhibits RPW8.2's EHM-targeting and its defense function, providing unequivocal evidence that EHM-localization of RPW8.2 is essential for activation of resistance to powdery mildew.

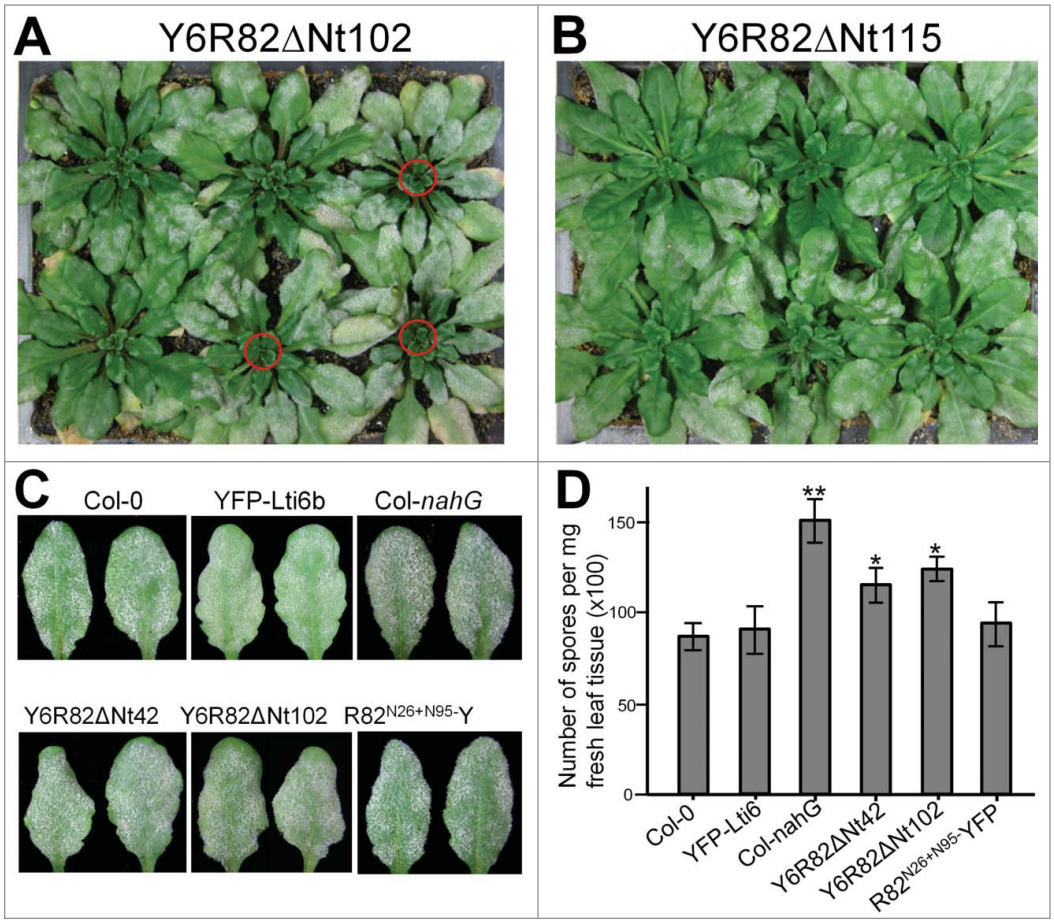

Figure 2. Expression of YFP-Lti6b–tagged RPW8.2 variants results in enhanced disease susceptibility. Six week-old plants were inoculated with *Gc* UCSC1 and disease phenotypes were assessed at 10 dpi. (**A**) Representative T1 plants transgenic for Y6R82ΔNt102 (as an example). Plants with enhanced disease susceptibility ('eds') are indicated by red circles. (**B**) Representative T1 plants transgenic for Y6R82ΔNt115 (as an example). No plants with apparent 'eds' were seen. (**C**) Representative individual infected leaves of indicated genotypes. Col-*nahG* is SA-deficient and displays 'eds'. (**D**) Quantification of disease susceptibility. Data represent means ± SE from one of 3 independent experiments. Student's *t*-test was used to compare value of other genotypes to that of Col-0. $*P < 0.05$; $**P < 0.001$.

Discussion

In this study, we unexpectedly found that expression of several nonfunctional, YFP-Lti6b–tagged mutant RPW8.2 proteins resulted in 'eds' to powdery mildew in the absence of *RPW8* and largely abrogated RPW8-mediated resistance. Since expression of those that show EHM-oriented docking produced a more obvious dominant-negative effect, our observations (i) suggest the existence of additional EHM-based defense proteins other than RPW8 in Arabidopsis and (ii) highlight the importance of a "healthy" smooth EHM-oriented trafficking pathway for mounting host defenses against powdery mildew pathogens.

What proteins might function at the EHM to confer basal resistance to powdery mildew in Arabidopsis accession Col-0 that lacks *RPW8.1* and *RPW8.2*? Given that Col-0 contains 4 homologs of *RPW8*, namely *HR1*, *HR2*, *HR3* and *HR4* at the same ~12Kb region where *RPW8* is located,[7,19] it is reasonable to speculate that these RPW8 homologous proteins might also be EHM-localized defense proteins (albeit their defense activities are probably much lower compared to RPW8.2, accounting for Col-0's susceptibility to *Gc* UCSC1). Hence, expression of "poisonous" Y6R82ΔNt102 might also interfere EHM-targeting of these RPW8 homologous proteins, resulting in loss of their function in basal resistance. If so, this might have circumvented the difficulty in assessing the likely redundant function of these genes through conventional genetic approaches (since these genes are tightly linked and have been under purifying selection[19]), thereby providing genetic evidence for their role in haustorium-targeted defense in Arabidopsis. Apparently, to substantiate this inference, we will need to examine the subcellular localization of these RPW8 homologs in Col-0 wild-type and their EHM-targeting efficiency in Col-0 lines expressing Y6R82ΔNt102 in our future experiments.

The dominant negative effect of Y6R82ΔNt102 might in part be attributable to its competition for the same set of trafficking components such as SNAREs or Rab proteins recruited for targeting EHM resident proteins. Consistent with this idea, a recent

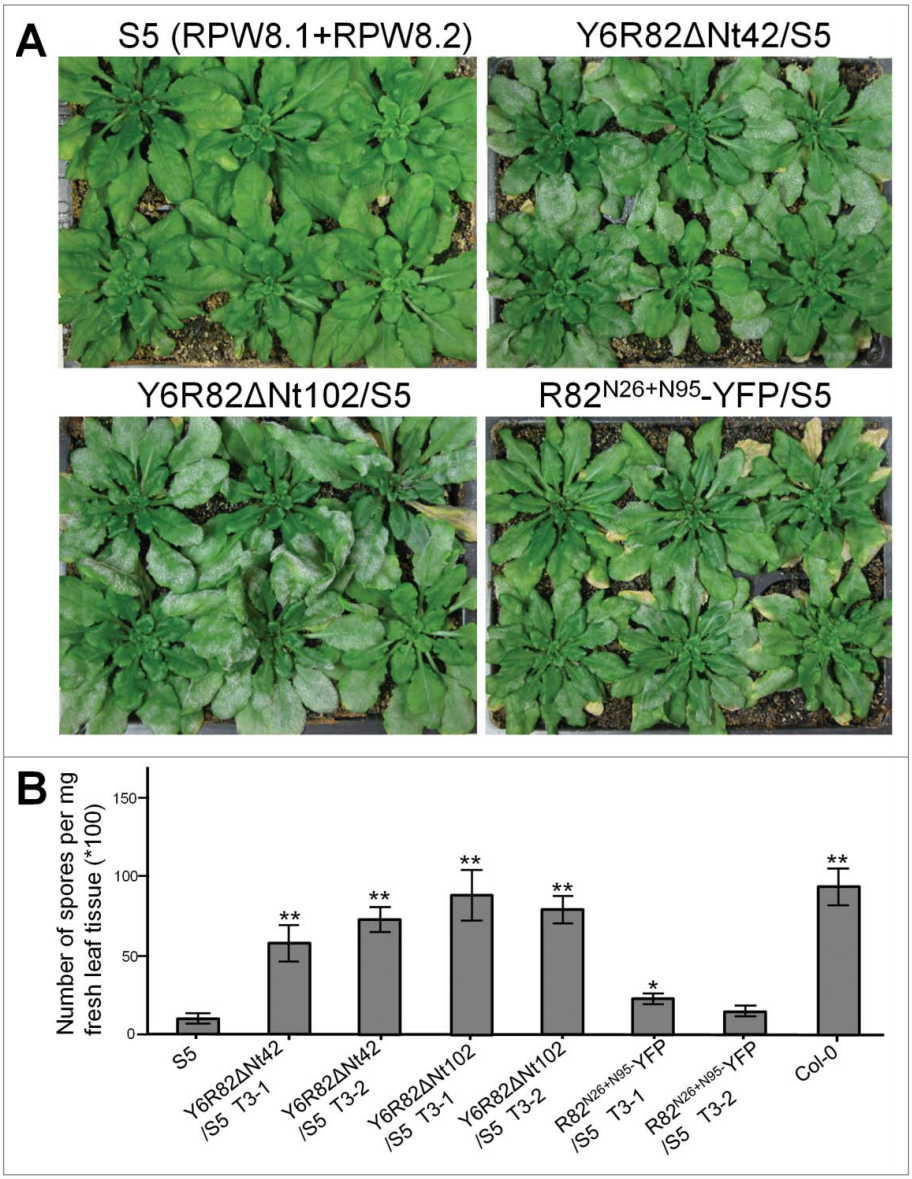

Figure 3. Expression of YFP-Lti6b–tagged RPW8.2 variants abrogates RPW8-mediated resistance. Six week-old plants were inoculated with *Gc* UCSC1 and disease phenotypes were assessed at 10 dpi. (**A**) Disease phenotypes of S5 (expressing both *RPW8.1* and *RPW8.2*), and S5 lines transgenic for indicated DNA constructs. Note the differences in the amount of whitish powdery mildew in different trays. (**B**) Quantification of number of spores per mg fresh leaves for the indicated genotypes. Data represent means ± SE from one of 3 independent experiments. Student's *t*-test was used to compare value of other genotypes to that of S5. $*P < 0.05$; $**P < 0.001$.

report showed that 2 homologous v-SNAREs Vamp721 and Vamp722 are also engaged in targeting RPW8.2-YFP to the EHM[20] in addition to their role in the default secretory pathway and the polarized trafficking to the fungal penetration site.[21] In most cases, genetic disruption of these conserved trafficking components results in severe deleterious effect in plant development,[22,23] which often hinders characterization of their role in a specific trafficking pathway. A dominant negative cargo protein like Y6R82ΔNt102 is a valuable tool in assessing the role of polar transport of the target cargo protein(s) and the engaged trafficking pathway in a specific biological process (i.e., haustorium-targeted resistance in the case of RPW8.2), as it would less likely affect other trafficking pathways. Consistent with this notion, we did not observe any developmental defect in the transgenic plants expressing Y6R82ΔNt102.

Since we did not detect obvious dominant negative effect from expression of YFP-RPW8.2 (which is defective in defense but retains proper EHM-localization[13] (not shown), or YFP-Lti6b (**Fig. 2C–2D**), it is reasonable to speculate that inserting Lti6b between YFP and RPW8.2 may change the overall membrane topology of RPW8.2, making the fusion protein neither functional in defense nor capable of fusing with the EHM, yet "poisonous" to functional RPW8.2 and perhaps other RPW8 family members when incorporated into the EHM-oriented trafficking pathway. Given that expression of the non-EHM–localized $R82^{N26+N95}$-YFP has no or little effect on RPW8-medated resistance, one might think that Y6R82ΔNt102 may reside in the same vesicles along with functional RPW8.2 thereby interfering with its normal vesicle traffic and fusion with the EHM. This agrees well with our observations that RPW8.2-RFP's EHM-targeting efficiency was more significantly reduced in Col-0 lines co-expressing Y6R82ΔNt102 than lines co-expressing $R82^{N26+N95}$-YFP (**Fig. 4C**). However, due to the fact that we rarely observed punctate vesicles for RPW8.2-RFP along the trafficking pathway (implying that the properties of RFP are different from those of YFP in manifestation of fluorescence signal), we were unable to examine if Y6R82ΔNt102 colocalizes with RPW8.2-RFP in punctate vesicles and interferes with its EHM-targeting. Hence, we cannot formally exclude other possibilities such as transgene-induced silencing of RPW8.2 for the loss of RPW8.2-mediated resistance in the mildew-susceptible S5 plants expressing Y6R82ΔNt102.

EHM docking of YFP-Lti6b–tagged mutant RPW8.2 proteins is difficult to explain, given that Y6R82ΔNt102, which contains neither of the 2 defined EHM-targeting motifs,[14] exhibited better EHM-docking than Y6R82ΔNt22, which contains both EHM-targeting motifs. It is possible that the C-terminal tail of RPW8.2 contains a cryptic EHM-targeting signal (and if so,

amino acids from 103 to 115 of RPW8.2 may be critical since removal of these 13 amino acid in Y6R82ΔNt115 resulted in loss of EHM-docking; **Table 1**) that may come to effect when the 2 proper EHM-targeting motifs are removed. Another possibility is that the EHM-oriented trafficking pathway is very active in haustorium–invaded cells during EHM biogenesis, and certain membrane proteins such as Y6R82ΔNt102 can more readily enter this pathway, and get passively docked to the EHM. The partial or complete exclusion of other YFP-Lti6b–tagged RPW8.2 fusion proteins from entering this pathway suggests that certain restrictions may apply with regard to what membrane protein cargos that can enter this pathway.

Our earlier studies indicated that the 2 functional arms of RPW8.2 (i.e., defense activation and EHM-localization) are under control of separate mechanisms. We speculated that integration of these 2 arms is key to activation of RPW8-mediated broad-spectrum resistance.[9,13] Results from this study fully support this inference, since expression of Y6R82ΔNt102 affects both the defense and EHM-localization of wild-type RPW8.2. How exactly Y6R82ΔNt102 affects RPW8.2′s defense function is not clear. One possibility is that Y6R82ΔNt102 may disrupt RPW8.2′s interaction with other defense-related proteins such as 14-3-3lambda or PAPP2C (a protein phosphatase type 2C)[24,25] at a specific stage of RPW8.2 vesicle transport or at the EHM. Notably, expression of Y6R82ΔNt102 almost completely abolished powdery mildew resistance of S5 plants expressing both *RPW8.1* and *RPW8.2* (**Fig. 3**). This result seems to suggest that RPW8.1′s defense function is also affected despite that RPW8.1 is normally expressed in mesophyll cells and functions either additively or co-operatively with RPW8.2 in the epidermal cells where haustoria invade.[10] One possible explanation is that expression of Y6R82ΔNt102 from the *RPW8.2* promoter in mesophyll cells may also interfere with RPW8.1′s localization to the periphery of chloroplasts, [8] thereby also affecting its defense function. Alternatively, functional interference of HR1, HR2, HR3 and HR4 in the S5 background by Y6R82ΔNt102 may also in part account for the loss of resistance in S5 plants.

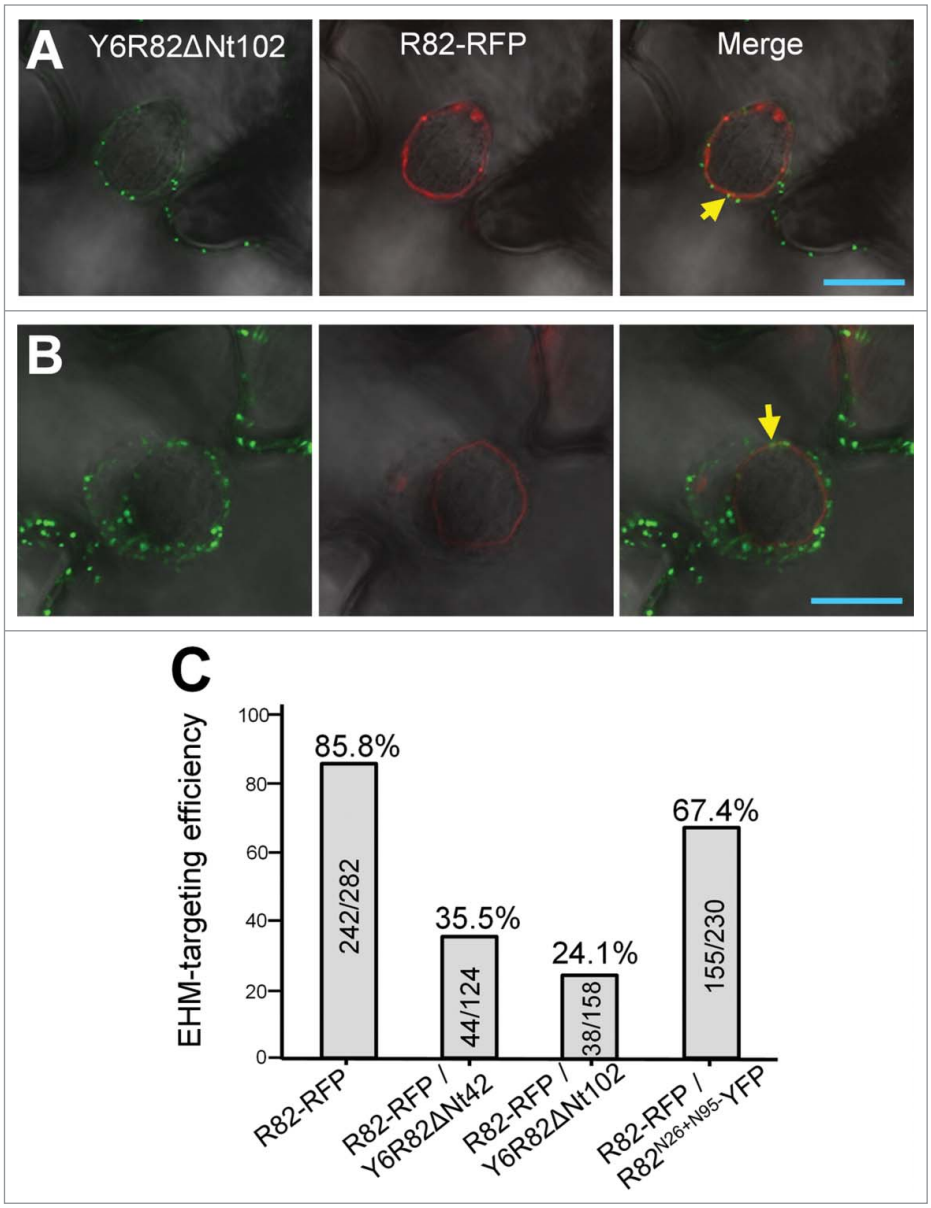

Figure 4. Expression of YFP-Lti6b–tagged RPW8.2 variants compromises EHM-targeting of RPW8.2-RFP. (**A**–**B**) Representative confocal image (single optical section) showing that strong RFP signal from RPW8.2-RFP at the EHM often coincided with no or weak YFP signal from Y6R82ΔNt102 around the EHM (**A**) or that strong YFP signal in puncta docked around the EHM often correlated with no or weak RFP signal from RPW8.2-RFP at the EHM (**B**). Bar represents 10 μm. (**C**) Quantification of EHM targeting efficiency in leaf epidermal cells of plants with indicated genotypes. Haustoria were visualized by propidium iodide staining. EHM targeting efficiency of RPW8.2-RFP was calculated as the percentage of haustoria with RFP–labeled EHM vs. total haustoria using the numbers in the columns.

In summary, our results from this study provide genetic and cell biological evidence for the importance of a smooth EHM-oriented trafficking pathway for activating haustorium-targeted resistance against powdery mildew and imply that EHM resident proteins other than RPW8 may be engaged in basal resistance in susceptible Arabidopsis plants.

Materials and Methods

Plants growth and transformation

Seeds of *Arabidopsis thaliana* were sown in Metromix 360 (Maryland Plant and Suppliers, USA) and cold treated (4°C for 3 days), and seedlings were kept under 22°C, 75% relative humidity, short-day (8 h light at ~125 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 16 h dark) conditions for 5 to 6 weeks before pathogen inoculation or other treatments.

Arabidopsis thaliana accession Col-0 (or Col-*gl* carrying the glabrous mutation) was used for generation of all transgenic lines described in this study via conventional Agrobacterium-mediated stable transformation. Unless otherwise indicated, at least 24 independent T1 transgenic lines were generated and tested with powdery mildew.

DNA constructs

The methods used for cloning the DNA constructs under control of the *RPW8.2* promoter (see **Table 1**) were essentially the same as previously described.[9,14] Briefly, the *RPW8.2* native promoter was amplified with primers EcoR82PF (5′-CAGAATTCACCGAAATTGTTAGTATTCA-3′) and BamR82PR (5′-ATGGATCCGAAATTAGTTTGTTAGCTCTCGAG-3′), and cloned into pPZP211 leading to the vector pPR82R5. YFP was amplified with primers BglYFPF1(5′-TCAGATCTATGGTGAGCAAGGGCGAG-3′) and BamYFPR1(5′-TGGGATCCGACTTGTACAGCTCGTCCATG-3′) and cloned into pPR82R5 resulting in the vector pP2BglYFP. Lti6b was amplified using LTI6btpF (5′-CACCAGATCTTAGTACAGCCACTTTCGTAGAGATTA-3′) and BamLTI6bR (5′-CAGGATCCCTTGGTGATGATATAAAGAGCGTAA-3′) primer pairs, and cloned into pP2BglYFP generating the vector pY6B. RPW8.2 fragments with desired N-terminal truncations were amplified by a high-fidelity thermostable DNA polymerase with appropriate forward primers (Supplemental **Table 2** of Wang et al. 2013) and reverse primer BglR823′R (5′-TGAGATCTTTTGTTGTTTTTTACTCT-3′), and cloned into the *Bam*HI site of pY6B containing the 3′-*UTR* of *RPW8.2*.

Pathogen infection and microscopy

Fresh mature spores of powdery mildew isolate *Golovinomyces cichoracearum* (*Gc*) UCSC1 were evenly inoculated on leaves of 6 week-old Arabidopsis plants and disease reaction phenotypes were assessed using methods as previously described.[16] Laser scanning confocal microscopy images were acquired as previously described[9,14] by using the Zeiss LSM 710 microscope. The image data were processed using Zeiss ZEN microscope software (2012 edition) and Adobe Photoshop CS4.

Phenotypic evaluation and microscopic examination were done with all T1 lines for each DNA construct, and the results were confirmed with T2 or T3 generations for some selected T1 lines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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