Arabidopsis ARF-GTP exchange factor, GNOM, mediates transport required for innate immunity and focal accumulation of syntaxin PEN1

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Edited by David C. Baulcombe, University of Cambridge, Cambridge, United Kingdom, and approved June 6, 2012 (received for review October 26, 2011)

Penetration resistance to powdery mildew fungi, conferred by localized cell wall appositions (papillae), is one of the best-studied processes in plant innate immunity. The syntaxin PENETRATION (PEN)1 is required for timely appearance of papillae, which contain callose and extracellular membrane material, as well as PEN1 itself. Appearance of membrane material in papillae suggests secretion of exosomes. These are potentially derived from multivesicular bodies (MVBs), supported by our observation that ARA6-labeled organelles assemble at the fungal attack site. However, the trafficking components that mediate delivery of extracellular membrane material are unknown. Here, we show that the delivery is independent of PEN1 function. Instead, we find that application of brefeldin (BF)A blocks the papillary accumulation of GFP-PEN1–labeled extracellular membrane and callose, while impeding penetration resistance. We subsequently provide evidence indicating that the responsible BFA-sensitive ADP ribosylation factor–GTP exchange factor (ARF-GEF) is GNOM. Firstly, analysis of the transheterozygote *gnom*$^{B4049/emb30\text{-}1}$ (*gnom*$^{B/E}$) mutant revealed a delay in papilla formation and reduced penetration resistance. Furthermore, a BFA-resistant version of GNOM restored the BFA-sensitive papillary accumulation of GFP-PEN1 and callose. Our data, therefore, provide a link between GNOM and disease resistance. We suggest that papilla formation requires rapid reorganization of material from the plasma membrane mediated by GNOM. The papilla material is subsequently presumed to be sorted into MVBs and directed to the site of fungal attack, rendering the epidermal plant cell inaccessible for the invading powdery mildew fungus.

fungal penetration | vesicle transport | GTPase | defense | pathogen

Plants depend on innate immunity to defend themselves against potentially infectious pathogens such as viruses, bacteria, and fungi. One type of innate immunity is penetration resistance, where the plant executes a timely defense response to effectively hinder fungi from entering the plant cell. In numerous studies of cereals attacked by powdery mildew fungi, penetration resistance has been associated with a papilla response, where a dome-shaped cell wall apposition is deposited by the epidermal cell between the cell wall and plasma membrane (PM) (1). Whereas *Arabidopsis* is a host for certain powdery mildew species, including *Golovinomyces cichoracearum*, it is a nonhost for *Blumeria graminis* f.sp. *hordei*, the powdery mildew pathogen of barley. However, *B. graminis* f.sp. *hordei* can germinate on *Arabidopsis* and occasionally succeeds in entering the epidermal cell, but it cannot proliferate.

In genetic screens for mutations that increase penetration by *B. graminis* f.sp. *hordei* in *Arabidopsis* nonhost plants, three *PENETRATION* (*PEN*) genes have been identified, representing two separate pathways. One pathway is represented by the two genes, *PEN2* and *PEN3*, encoding a β-thioglucoside glucohydrolase and an ATP-binding cassette (ABC) transporter, respectively. These two enzymes are believed to generate and transport glucosinolates to the site of pathogen attack (2–5). The second penetration-resistance pathway requires *PEN1*. This gene encodes the PM-syntaxin, syntaxin of plants (SYP)121, and, together with soluble *N*-ethylmaleimide-sensitive factor (NSF) adaptor protein (SNAP)33 and vesicle-associated membrane protein (VAMP)721/VAMP722, it forms PM-localized ternary SNARE complexes required for penetration resistance (6, 7). Interestingly, the knockout mutant, *pen1-1*, is developmentally indistinguishable from wild-type plants, whereas *snap33-1* and the double mutant *vamp721-1/vamp722-1* are severely retarded in their growth (7, 8). Although this underlines the requirement for vesicle transport in pathogen defense, it also suggests that the PEN1-dependent penetration-resistance pathway shares trafficking components that are vital for general development. This SNARE-mediated defense mechanism is conserved between monocotyledons and dicotyledons, because the PEN1 ortholog, required for mlo-specified resistance (ROR)2, is required for penetration resistance in barley (6). By using fluorescently labeled protein fusions, both PEN1 and ROR2 were found to strongly accumulate in the papilla (9, 10). Here, PEN1 colocalizes with the lipophilic dye FM4-64 outside the PM, suggesting that it is secreted on exosomes (11). These have been suggested to be derived from multivesicular bodies (MVBs) (11), in agreement with previous findings where MVBs were in close proximity to the papilla and small vesicles were embedded in the papillary matrix (12). Recently, we identified the barley ADP ribosylation factor (ARF)A1b/1c GTPase that appeared to be linked to the function of MVBs. ARFA1b/1c was both required for ROR2-dependent penetration resistance, as well as ROR2 and callose deposition in papillae (13). This indicates that MVBs are vital for penetration resistance. However, although relocalization of ROR2 and PEN1 may involve MVB formation, little is known about the trafficking components that mediate this transport.

Here, we show that the secretion of extracellular membrane material follows callose and PEN1 accumulation into the papillary matrix. Interestingly, this secretion does not require PEN1 function. Instead, we find that the large ARF–GTP exchange factor (ARF-GEF) GNOM is vital for accumulation of callose and GFP-PEN1 in the papillary matrix. Based on our findings,

Author contributions: M.E.N., A.F., and H.T.-C. designed research; M.E.N., A.F., and T.U. performed research; T.U. contributed new reagents/analytic tools; M.E.N., A.F., and H.B. analyzed data; and M.E.N. and H.T.-C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1117596109/-/DCSupplemental.

we suggest that papilla formation requires a GNOM-mediated trafficking pathway to recycle preexisting PM material to the papilla, which ultimately leads to penetration resistance.

Results

GFP-PEN1 Is a Marker for Papillary Extracellular Membrane. The previous indication of GFP-PEN1–positive exosomes present in the papillary matrix suggested that secretion of intraluminal vesicles of MVBs is an integral part of papilla formation (11). To analyze whether PEN1 itself is required for releasing membrane into papillae, we made use of the fluorescent dye FM4-64 that stains the lipophilic portion of membrane components. Although FM4-64 is most often used to study endocytic pathways in plant cells, its staining properties allow visualization of membrane material in papillae. At 23 h post inoculation (hpi) with *B. graminis* f.sp. *hordei*, FM4-64 was infiltrated into *Arabidopsis* leaves and left to incorporate into membranes for 1 h. As previously described by Meyer et al. (11), *pen1-1* plants expressing GFP-PEN1 showed colocalization of GFP-PEN1 and FM4-64 in both the PM and preformed papillae (Fig. 1*A* and Fig. S1*A*). The closest homolog of PEN1 is the PM-localized SYP122. The double mutant *pen1-1 syp122-1* is severely dwarfed. Overexpression of YFP-SYP122 rescued this dwarfed phenotype but did not rescue penetration resistance (Fig. S1 *B–D*). Therefore, this normally functioning YFP-SYP122 is a useful alternative PM-restricted marker. Interestingly, YFP-SYP122, expressed in *pen1-1 syp122-1* plants, was restricted to the margin of the papilla and did not fluoresce across the papilla body (Fig. 1*B*). By visualizing the PM around the papilla in this way, the extracellular deposition of GFP-PEN1 became apparent. The presence of extracellular membrane was found to be independent of PEN1, because FM4-64 stained the papillary matrix outside of the PM in these mutant plants, despite the absence of PEN1 function. The FM4-64 staining of preformed papillae and accumulation of GFP-PEN1 did not enable us to discriminate between recycled and de novo–synthesized papilla material. Attempts to follow the event of FM4-64 internalization during *B. graminis* f.sp. *hordei* attack failed, because we did not succeed in washing out excess FM4-64 from the epidermal cell apoplast before papilla formation. Moreover, use of cycloheximide (CHX), an inhibitor of de novo protein synthesis, was found to interfere with spore germination. Therefore, the origin of the papilla material was unresolved at this point. Nonetheless, these observations support that the membrane protein GFP-PEN1 could be secreted on exosomes into the papillary matrix and that we can track extracellular membrane using GFP-PEN1. The PEN1-independent release of membrane into papillae was further confirmed by FM4-64 staining in *B. graminis* f.sp. *hordei*–attacked *pen1-1* mutant plants (Fig. S2 *A–D*).

Although the origin and nature of extracellular membrane is poorly understood in plants, it is likely to require MVB fusion and secretion of exosomes, as discussed above. We, therefore, observed the localization of the two Rab5-like GTPases, ARA6 and ARA7, during attack by *B. graminis* f.sp. *hordei*. These proteins are commonly used MVB markers (14). Plants expressing either ARA6-GFP or GFP-ARA7 were monitored after inoculation. Only in the case of ARA6-GFP did we find that the GFP-labeled organelles accumulated near the fungal-attack site (Fig. 1 *C* and *D* and Fig. S3 *A* and *B*). This finding supports previous descriptions of MVBs accumulating near attack sites and may suggest a delivery track of GFP-PEN1–positive membrane material into the papilla (12, 15).

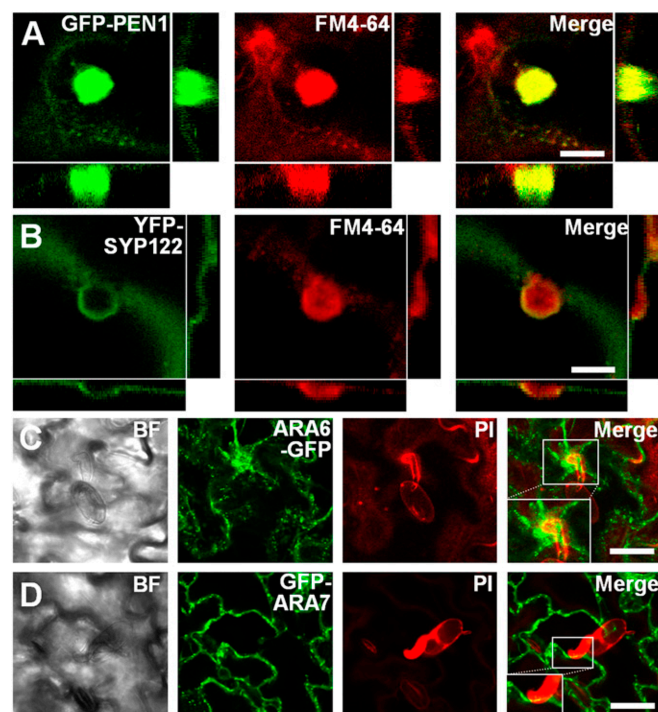

Fig. 1. *B. graminis* f.sp. *hordei* induces accumulation of GFP-PEN1 and membrane material outside the PM and ARA6-GFP near the site of fungal attack. (*A* and *B*) Leaves of *pen1-1* (*A*) and *pen1-1 syp122-1* (*B*) expressing functional GFP-PEN1 and YFP-SYP122, respectively, were stained with FM4-64 and monitored at 24 hpi with *B. graminis* f.sp. *hordei*. Each image shows *Z*-stack maximum projections enabling side views of the fungal attack site. (*C* and *D*) Plants expressing ARA6-GFP (*C*) and GFP-ARA7 (*D*) 24 hpi with *B. graminis* f.sp. *hordei* (red). [Scale bars: 5 μm (*A* and *B*); 10 μm (*C* and *D*).]

BFA Breaks Penetration Resistance. To identify other membrane-trafficking components that are involved in PEN1-dependent penetration resistance and potentially in exosome secretion into papillae, we analyzed the effects of the vesicle-trafficking inhibitor brefeldin (BF)A, which blocks the action of certain ARF-GEFs (16). Therefore, leaves were infiltrated with BFA 3 h before *B. graminis* f.sp. *hordei* inoculation and stained with trypan blue 48 hpi. Analysis of this material revealed that BFA significantly reduced penetration resistance (Fig. 2*A*). Meanwhile, penetration resistance in *pen1-1* was not affected, suggesting that BFA inhibits the PEN1-dependent pathway, which is already blocked in the mutant. However, a similar result was obtained when applying BFA to *pen2-1* (Fig. 2*A*), suggesting the effect of BFA is not specific for the PEN1-dependent pathway. It should be noted that the *pen1-1 pen2-1* double mutant has an increased penetration rate relative to single mutants (3). This indicates that the two pathways are separate and also shows that the penetration rate of *B. graminis* f.sp. *hordei* on *pen1-1* does not represent a maximum penetration level. Reduced concentrations of BFA (50 μM), applied by petiole dipping from 24 h before *B. graminis* f.sp. *hordei* inoculation, had a similar effect on wild-type plants. After BFA treatments, we observed normal development of haustoria and secondary hyphae of *B. graminis* f.sp. *hordei*, as well as of the *Arabidopsis*-virulent powdery mildew fungus, *G. cichoracearum*. This indicated that BFA had no impact on fungal growth per se and that the treated epidermal cells are fully capable of sustaining fungal growth.

BFA Blocks Accumulation of GFP-PEN1 and Callose in Papillae. Next, we wanted to investigate how BFA influenced papilla localization of GFP-PEN1 upon attack by *B. graminis* f.sp. *hordei*. Leaves of *pen1-1* plants expressing GFP-PEN1 were treated as described above but analyzed at 24 hpi. In leaves treated with BFA, GFP-PEN1 accumulation at the sites of attempted *B. graminis* f.sp. *hordei* penetration was often absent (Fig. 2 *B* and *C* and Fig. S4).

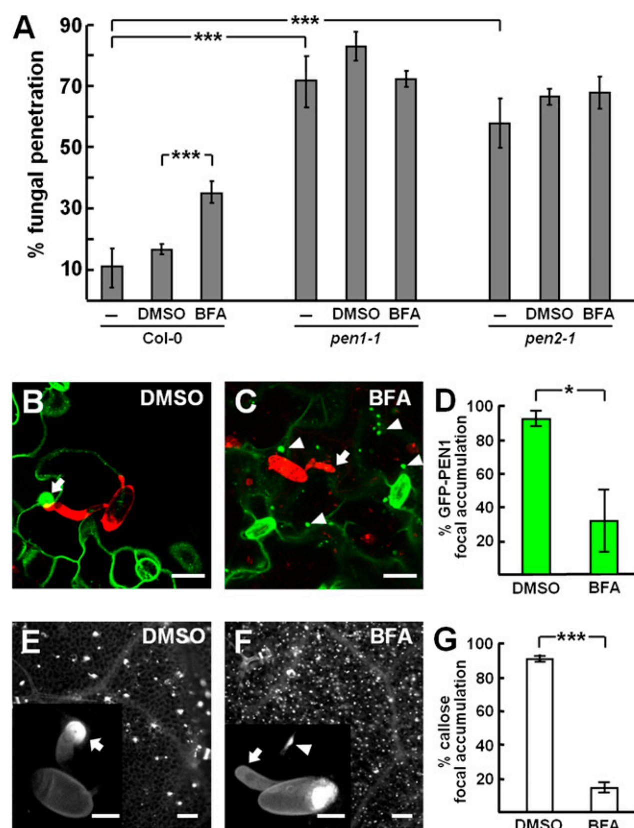

Fig. 2. BFA hinders penetration resistance and blocks fungal induced papilla responses. (*A*) Leaves of Col-0, *pen1-1*, or *pen2-1* were infiltrated with either DMSO or BFA (300 μM) in DMSO or left untreated for 3 h before inoculation with *B. graminis* f.sp. *hordei*. The frequency of fungal penetration was analyzed at 48 hpi. (*B–G*) Leaves of *pen1-1* expressing GFP-PEN1 analyzed for GFP accumulation (*B–D*) or Col-0 analyzed for callose accumulation (*E–G*) at 24 hpi. (*B–F*) DMSO control treatments (*B* and *E*) and BFA treatments (*C* and *F*). (*B, C, E*, and *F*) Focal accumulations dependent on *B. graminis* f.sp. *hordei* (arrow) and independent of *B. graminis* f.sp. *hordei* (arrowheads). (*D* and *G*) Frequency of GFP-PEN1 (*D*) and callose (*G*) accumulation at fungal attack sites after BFA treatment. (*A, D*, and *G*) Each data point is represented as mean. [Error bars represent SD ($n = 4$).] $*P < 0.05$; $***P < 0.001$. For each leaf (repeat), a minimum of 60 germinated spores were scored. [Scale bars: 10 μm (*B* and *C*); 100 μm (*E* and *F*); 5 μm (*Insets*).]

The frequency of normal papillary GFP-PEN1 accumulation was reduced from 92% to 32% after BFA treatment (Fig. 2*D*). Consistently, no FM4-64 accumulation was observed in papillae in leaves treated with BFA (Fig. S4 *C* and *D*). Meanwhile, BFA induced small endosomal aggregates of GFP-PEN1 in all epidermal cells, irrespective of *B. graminis* f.sp. *hordei* attack (Fig. 2*C* and Fig. S4*B*). Similar aggregates are known as BFA bodies (or compartments) in *Arabidopsis* root and meristem cells (17, 18). In leaves treated for 1 h with BFA and CHX, GFP-PEN1 was found to colocalize with FM4-64 in the BFA-body-like structures (Fig. S5). This suggested that the GFP-PEN1 accumulating in these bodies is not de novo–synthesized, but rather derived from continuous recycling from the PM. Finding that BFA effectively inhibits accumulation of GFP-PEN1 at the site of attempted *B. graminis* f.sp. *hordei* penetration prompted us to investigate whether BFA also affects the focal accumulation of PEN2-GFP (2). However, in contrast to GFP-PEN1, we did not detect any differences in the localization of PEN2-GFP after BFA treatment (Fig. S6).

Another well-known constituent of papillae is the β-1,3-glucan, callose. We monitored the accumulation of callose at the penetration site and found that BFA prevented accumulation of this polymer as well (Fig. 2 *E–G*). Interestingly, BFA also induced callose depositions independently of *B. graminis* f.sp. *hordei* attack. These depositions occurred at the cell periphery of both epidermal and mesophyll cells and could be detected as early as 2 h after injection of BFA. The callose depositions were distinct from the GFP-PEN1 containing BFA bodies, described above (Fig. S7).

GNOM Is Required for Penetration Resistance, Papillary GFP-PEN1, and Callose Accumulation. Because BFA affects penetration resistance, as well as GFP-PEN1 and callose accumulation, we turned to identify the assumed PEN1-pathway–associated vesicle-trafficking component targeted by this compound. BFA inhibits certain ARF-GEFs, indicating that one or more of these proteins are vital for penetration resistance. Firstly, we investigated the BFA-sensitive ARF-GEF *Arabidopsis thaliana* HopM interactor (*At*MIN)7 which has previously been associated with pathogen defense, including callose deposition (19). However, penetration resistance in *Atmin7* knockout mutant plants was comparable to that of wild-type plants, implying that *At*MIN7 is not vital for penetration resistance (Fig. S8). An alternative and well-studied BFA-sensitive ARF-GEF is GNOM (16, 20). Strong mutant alleles of GNOM result in severely dwarfed and stunted plants that cannot be scored for penetration resistance. Therefore, we analyzed $gnom^{B4049/emb30-1}$ ($gnom^{B/E}$) transheterozygote plants in which the two mutant proteins of the ARF-GEF dimer partially complement the nonfunctional domains of each other. These plants have a weaker phenotype, being slightly dwarfed with narrow, curled-down rosette leaves (21). $gnom^{B/E}$ displayed a significant increase in the percentage of penetration by *B. graminis* f.sp. *hordei*, demonstrating that GNOM is required for full penetration resistance (Fig. 3*A*). At 24 hpi, papillary callose accumulation in $gnom^{B/E}$ was indistinguishable from that of wild-type plants. However, a time-course study revealed that $gnom^{B/E}$ had an

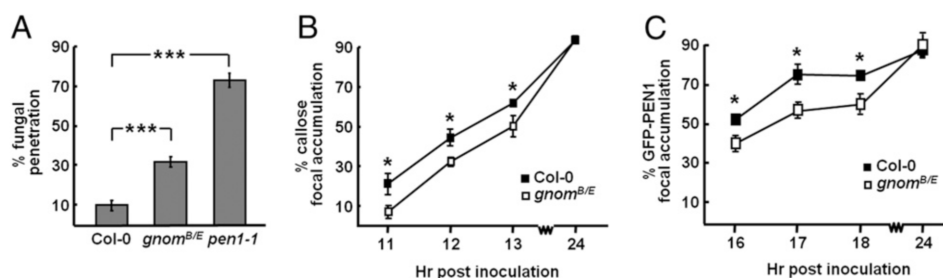

Fig. 3. Fully functional GNOM is required for penetration resistance and timely papilla response. (*A*) Frequency of fungal penetration analyzed at 48 hpi in Col-0, *pen1-1*, and $gnom^{B/E}$ leaves. (*B* and *C*) Frequency of callose (*B*) and GFP-PEN1 (*C*) accumulation at *B. graminis* f.sp. *hordei* attack sites in Col-0 and $gnom^{B/E}$. Each data point is represented as mean. [Error bars represent SD ($n = 4$).] $*P < 0.05$; $***P < 0.001$. For each leaf (repeat), a minimum of 60 germinated spores were scored.

approximate 30-min delay in the appearance of callose (Fig. 3*B*). A similar delay in callose deposition was described previously for *pen1-1* (9). GFP-PEN1 exhibited a comparable accumulation pattern in *gnom*$^{B/E}$. At 24 hpi, wild type and *gnom*$^{B/E}$ showed indistinguishable GFP-PEN1 deposition in the papillary matrix, whereas a delay was seen in *gnom*$^{B/E}$ at earlier time points (Fig. 3*C* and Fig. S9). The latter provides an indication of a mechanistic link between penetration resistance and papillary accumulation of extracellular membrane.

GNOM^{M696L} Rescues BFA Sensitivity of Papillary GFP-PEN1 and Callose Accumulation. Having seen that GNOM is required for full penetration resistance, we wanted to analyze whether it also is the BFA-targeted ARF-GEF that is associated with penetration resistance. Therefore, we crossed a transgene that was reported previously to encode a fully functional, but BFA-resistant, version of GNOM (GNOM^{M696L}) into GFP-PEN1–expressing plants. *GNOM*^{M696L} has been used previously to study BFA effects on the relocalization of auxin-transport components that also accumulate in BFA bodies (17). In *gnom*$^{emb30-1}$ plants expressing GNOM^{M696L}, we found that the *B. graminis* f.sp. *hordei*–induced papillary accumulation of GFP-PEN1 and callose, normally blocked by BFA, is fully rescued at 24 hpi (Fig. 4 *A*–*F* and Fig. S10 *A*–*D*). This demonstrates that the BFA-sensitive ARF-GEF required for GFP-PEN1 and callose delivery to the papillary matrix is GNOM. Furthermore, the rescue of papilla formation shows that BFA treatments did not cause unintentional side effects. In contrast, GFP-PEN1 was still found to accumulate in BFA bodies (Fig. 4*D*) in *gnom*$^{emb30-1}$ GNOM^{M696L}, indicating that the continuous *B. graminis* f.sp. *hordei*–independent recycling of PEN1 (see above) occurs independently of GNOM. In addition, BFA-induced callose depositions at the periphery of unattacked cells were also unaffected by the replacement of GNOM by GNOM^{M696L} (Fig. S10*D*).

Having found that GNOM^{M696L} rescues BFA-sensitive GFP-PEN1 and callose delivery to papillae, we sought to investigate whether GNOM^{M696L} also rescues BFA-sensitive penetration resistance. Surprisingly, we found that in *gnom*$^{emb30-1}$ plants expressing GNOM^{M696L}, penetration resistance against *B. graminis* f.sp. *hordei* was clearly reduced, and it was not further affected after treatment with BFA (Fig. 4*G*). To ensure that the altered penetration resistance in *gnom*$^{emb30-1}$/*GNOM*^{M696L} was not attributable to an insertion of the transgene into a penetration-related gene such as *PEN2*, we back-crossed the *GNOM*^{M696L} transgene into Columbia (Col)-0 and tested for penetration resistance in selected F_2 plants. Again, *gnom*$^{emb30-1}$/*GNOM*^{M696L} lines homozygous for the mutation and the transgene had a higher penetration frequency than wild type. Meanwhile, Col-0/*GNOM*^{M696L}, homozygous for the transgene, did not show defects in penetration resistance (Fig. S11*A*). This showed that *GNOM*^{M696L} is not inserted into a gene essential for penetration resistance. Furthermore, it showed that although GNOM^{M696L} rescued the developmental phenotypes of *gnom*$^{emb30-1}$ (17), it did not fully rescue penetration resistance. Combined, the results obtained using *gnom*$^{B/E}$ and *gnom*$^{emb30-1}$/*GNOM*^{M696L} demonstrate that GNOM is important for the execution of innate immunity in *Arabidopsis*. Furthermore, the fact that the penetration resistance in *gnom*$^{emb30-1}$/*GNOM*^{M696L} was not further affected by BFA treatment, suggested that no other BFA-sensitive component influenced this phenotype. We speculated whether the inability of

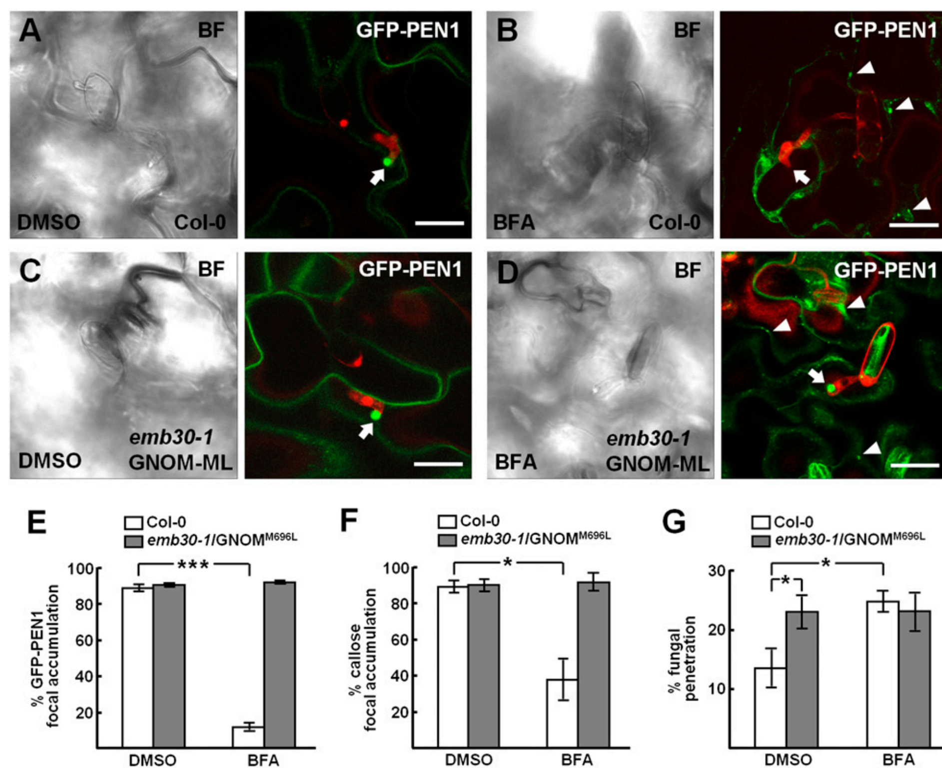

Fig. 4. GNOM^{M696L} confers BFA resistance of fungal-induced GFP-PEN1 and callose accumulation and hinders penetration resistance. (*A*–*D*) Papillary GFP-PEN1 accumulation (arrows) at 24 hpi after BFA treatment of Col-0 leaves (*A* and *B*) and *gnom*$^{emb30-1}$ leaves expressing GNOM^{M696L} (*C* and *D*). (*B* and *D*) Fungal-attack–independent focal GFP-PEN1 accumulations (arrowheads). (Scale bars: 10 μm.) (*E* and *F*) Frequency of GFP-PEN1 (*E*) and callose (*F*) accumulation at 24 hpi at *B. graminis* f.sp. *hordei* attack sites after BFA treatment of Col-0 leaves and *gnom*$^{emb30-1}$ leaves expressing GNOM^{M696L}. (*G*) Frequency of *B. graminis* f.sp. *hordei* entry after BFA treatment of Col-0 leaves and *gnom*$^{emb30-1}$ leaves expressing GNOM^{M696L}. Each data point is represented as mean. [Error bars represent SD ($n = 4$).] *$P < 0.05$; ***$P < 0.001$. For each leaf (repeat), a minimum of 60 germinated spores were scored. (Scale bars: 10 μm.)

$GNOM^{M696L}$ to rescue the penetration resistance could be attributable to a delayed papilla formation, as we found in the case of $gnom^{B/E}$. Based on previous experience (Fig. 3 *B* and *C*), we selected to quantify the callose deposition at 12 hpi and GPF-PEN1 focal accumulation at 17 hpi. The data could suggest a delayed papilla formation, because both markers showed slight reductions in $gnom^{emb30-1}/GNOM^{M696L}$ relative to wild-type Col-0, although only statistically significant in the case of GFP-PEN1 (Figs. S9 and S11 *B* and *C*).

Discussion

Since the discovery of PEN1, vesicle trafficking has been acknowledged as a vital part of plant innate immunity (6, 22). However, how PEN1 mediates resistance is still unknown. The observation of GFP-PEN1 within the papillary matrix suggests that PEN1 is secreted on exosomes (this work and ref. 11). This finding is supported by transmission electron microscopy images of barley papillae showing exosomes within the papillary matrix and clusters of MVBs in close proximity to the attack site (12). Secretion of papillary exosomes is likely to involve a direct fusion between MVBs and the PM, a fusion that potentially could implicate the action of PEN1 itself. In support of this, we recently reported that the plant-specific Rab5 homolog, the MVB-localized ARA6, facilitates SNARE interaction between the MVB-localized VAMP727 and PM-localized PEN1 (23). Finding that ARA6 accumulates in the vicinity of attack sites further supports that ARA6-positive MVBs deliver the papillary exosomes mediated by PEN1. However, in this present work, we show that the secretion of membrane into the papilla can occur in plants lacking PEN1, indicating that at least one other syntaxin is capable of mediating the proposed MVB-PM fusion. This agrees with previous observations where deposition of callose into the papilla is found to be delayed but not abolished in the absence of PEN1 SNARE complex formation (7, 9). Similarly, we predict that the secretion of membrane material into the papillary matrix is delayed in *pen1-1*.

Previously, it was thought that callose would be synthesized at the PM, directly into the papillary matrix. However, ultramicroscopic findings of callose within clathrin-coated pits at the PM and within MVBs suggest that callose, or components facilitating callose synthesis, are transported to the papilla via MVBs (24). In support of this, we found that treatment with BFA blocks not only the accumulation of GFP-PEN1 but also the deposition of callose into the papilla. This provides further support for the involvement of MVBs in transport of both PEN1-labeled exosomes and callose, or components facilitating callose synthesis, to the site of powdery mildew attack. In *Arabidopsis* roots, the secretory pathway is not affected by application of BFA, because of the insensitivity of GNOM-LIKE1, which mediates Golgi–endoplasmic reticulum (ER) trafficking (18, 25). Nonetheless, Langhans et al. (26) reported that 1/2-h treatments of 90 μM BFA could cause a redistribution of Golgi into the ER, suggesting that the secretory pathway in true leaves is more sensitive to BFA than in roots. In our long-term treatments (a minimum of 19 h), a block of the secretory pathway should, thus, lead to an ER accumulation of GFP-PEN1, as it has been reported previously for MYC-PEN1 (27), KNOLLE (28), and secretory GFP (25). However, we have not observed that GFP-PEN1 accumulates in the ER at any of the time points we have investigated, indicating that the secretory pathway is unaffected. The dependency of a functional secretory pathway for papillary accumulation of GFP-PEN1 and callose is, therefore, likely to be minimal. An alternative explanation is that papillae depend on reorganization of preformed material from elsewhere, such as the PM.

GNOM is a BFA-sensitive ARF-GEF, mediating recycling of endocytosed proteins back to the PM, a function required for the correct localization of auxin-efflux carriers (16). GNOM is, thus, vital for root gravitropism and development (17). Root hydrotropism, although mechanistically different, is also dependent on GNOM function (29, 30). Because GNOM is BFA-sensitive, we tested whether this ARF-GEF could be involved in penetration resistance. Analysis of the transheterozygote $gnom^{B/E}$ mutant revealed reduced penetration resistance linked to a delay in papilla formation. Moreover, these results were confirmed by our ability to rescue the BFA-induced inhibition of papillary GFP-PEN1 and callose using the BFA-insensitive $GNOM^{M696L}$. Combined, these observations provide a link between GNOM and disease resistance. Interestingly, GNOM functionality has been associated previously with MVBs in that a *gnom* mutant has been found to have abnormal MVBs (17). This is in line with our previous discovery that the depletion of barley ARFA1b/1c results in hampered penetration resistance and prevents accumulation of the barley ortholog of PEN1, ROR2, and callose into the papilla (13). It is striking that inhibition of GNOM in *Arabidopsis* and depletion of ARFA1b/1c in barley leads to the same set of phenotypes. This could indicate that a barley GNOM ortholog interacts directly with ARFA1b/1c.

The expression of the BFA-resistant $GNOM^{M696L}$ has been found previously to recover GNOM function completely, whereas $gnom^{B/E}$ shows reduced responses in general development, gravitropism, and hydrotropism (21). In our hands, $gnom^{emb30-1}$ expressing $GNOM^{M696L}$ was also developmentally indistinguishable from wild type and, furthermore, fully rescues the BFA-sensitive papilla formation when analyzed at 24 hpi. It was, therefore, surprising to find that $GNOM^{M696L}$ did not fully recover penetration resistance, a notion that might be explained by a slight delay in papillary GFP-PEN1 and callose accumulation. Although this indicates that the effects of BFA on papilla formation is attributable to specific inhibition of GNOM activity, we cannot completely exclude that another BFA-sensitive ARF-GEF is involved in the BFA inhibition of penetration resistance. However, we do find this unlikely based on our genetic evidence for GNOM being essential for penetration resistance and based on the fact that BFA did not affect this phenotype in $gnom^{emb30-1}/GNOM^{M696L}$.

Finding that GNOM is important for delivery of papilla material suggests to us that penetration resistance relies on fast reorganization of presynthesized material. Furthermore, the fact that the *pen1-1* mutation delays the papilla response implies that the correct timing of papilla formation is critical for penetration resistance. Logically, whether the papilla appears too late, or not at all, seems to have the same effect on the success of fungal entry. Interestingly, GNOM appears to be an evolutionarily distinct, plant-specific, ARF-GEF that plays a nonredundant and, thus, essential role in endosomal recycling (16). Likewise, the PEN1-interacting VAMP721 and VAMP722, also unique to plants, carry out functions that are important for penetration resistance (7). In addition, the recent discovery that the plant-unique ARA6 mediates PEN1-dependent membrane fusion between the PM and MVBs (23), both of which accumulate at the attack site (this work), could help to explain how papilla material is delivered to prevent fungal penetration. Combined, these observations indicate that plants use a unique set of membrane-trafficking components for the execution of penetration resistance.

Materials and Methods

Plant and Fungal Growth. *Arabidopsis thaliana* plants were grown at 21 °C, with 8 h of light at 125 microEinstein $s^{-1}m^{-2}$ per day. The barley powdery mildew fungus (*B. graminis* f.sp. *hordei*, isolate C15) was propagated on barley and the *Arabidopsis* powdery mildew fungus (*G. cichoracearum*, isolate UCSC1) was propagated on squash.

Inhibitor Treatment, Fungal Inoculation, and Scoring of Interactions. Chemicals were supplied from Sigma-Aldrich. BFA was kept as a 30 mM stock in DMSO, and CHX was kept as a 50 mM stock in EtOH. Inhibitors were further diluted in distilled water to achieve the working concentrations of 300 μM BFA and 50 μM CHX unless otherwise indicated. Leaves of 4-wk-old plants were infiltrated with the inhibitors or the proper DMSO or EtOH control solutions

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through the abaxial side of the leaf using a blunt syringe, 3 h before *B. graminis* f.sp. *hordei* inoculation. Alternatively, BFA was taken up by transpiration after dipping the leaf petiole in the solution from 24 h before *B. graminis* f.sp. *hordei* inoculation. In this case, solutions were replaced once a day. For scoring of penetration success, leaf material was trypan blue–stained at 48 h post *B. graminis* f.sp. *hordei* inoculation. Penetration was determined by the presence of a fungal haustorium using light microscopy. Callose staining was made using 0.01% aniline blue in 1 M glycine [NaOH (pH 9.5)] and visualized by UV epifluorescence. All experiments were repeated at least three times with similar results.

Fluorescent Protein, FM4-64, and Callose Detection Using Confocal Microscopy. *pen1-1* expressing the transgene *35S::GFP-PEN1* has been described previously and was used in all experiments (6). This transgenic line was used for all observations of GFP-PEN1 and, thus, either back-crossed to Col-0 or into $gnom^{B/E}$ or $gnom^{emb30\text{-}1}/GNOM^{M696L}$. The transgene construct *35S::YFP-SYP122*, generated in the pB7WGY2,0 vector (31), was transformed into *pen1-1 syp122-1*. For visualization of *B. graminis* f.sp. *hordei* spores, leaves were dipped in a solution of 2.5% mannitol/0.01% (vol/vol) silwet/0.2% (wt/vol) propidium iodide to stain fungal structures. Staining of membranes was achieved by syringe infiltration of 5 μM FM4-64, 1 h before examination. For colocalization studies of GFP and callose, samples were fixed overnight in a 2% formaldehyde solution and then rinsed in water and stained with aniline blue. Samples were examined using a 40× water immersion or dip-in lens mounted on a Leica CLSM TCS SP2 or TCS SP5 confocal microscope. Projections of serial confocal sections, image overlays, and contrast enhancement were performed using image processing software (Scion Image, Photoshop, or MetaVue 6.2).

ACKNOWLEDGMENTS. We thank Gerd Jürgens for providing mutant and transgenic lines and Frantisek Baluska for helpful suggestions. Imaging data were collected at the Center for Advanced Bioimaging (CAB) Denmark, University of Copenhagen. This work was supported by the Danish Agricultural and Veterinary Research Council; the Carlsberg Foundation, Denmark; the Danish Council for Independent Research/Technology and Production Sciences; and the Danish Council for Independent Research/Natural Sciences.

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