Interaction of the *Arabidopsis* GTPase RabA4c with Its Effector PMR4 Results in Complete Penetration Resistance to Powdery Mildew

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The (1,3)-β-glucan callose is a major component of cell wall thickenings in response to pathogen attack in plants. GTPases have been suggested to regulate pathogen-induced callose biosynthesis. To elucidate the regulation of callose biosynthesis in *Arabidopsis thaliana*, we screened microarray data and identified transcriptional upregulation of the GTPase RabA4c after biotic stress. We studied the function of RabA4c in its native and dominant negative (dn) isoform in *RabA4c* overexpression lines. RabA4c overexpression caused complete penetration resistance to the virulent powdery mildew *Golovinomyces cichoracearum* due to enhanced callose deposition at early time points of infection, which prevented fungal ingress into epidermal cells. By contrast, *RabA4c*(dn) overexpression did not increase callose deposition or penetration resistance. A cross of the resistant line with the *pmr4* disruption mutant lacking the stress-induced callose synthase PMR4 revealed that enhanced callose deposition and penetration resistance were PMR4-dependent. In live-cell imaging, tagged RabA4c was shown to localize at the plasma membrane prior to infection, which was broken in the *pmr4* disruption mutant background, with callose deposits at the site of attempted fungal penetration. Together with our interactions studies including yeast two-hybrid, pull-down, and in planta fluorescence resonance energy transfer assays, we concluded that RabA4c directly interacts with PMR4, which can be seen as an effector of this GTPase.

**INTRODUCTION**

The deposition of the (1,3)-β-glucan polymer callose is involved in several fundamental processes of plant development as well as plant defense. Regarding defense responses to pathogen attack, particular attention has been focused on the formation of cell wall thickenings. So-called papillae contain callose as the most common chemical constituent, but also proteins (e.g., peroxidases and thionins), phenolics, and other antimicrobial and antifungal constituents (Aist and Williams, 1971; Sargent et al., 1973; Mercer et al., 1974; Sherwood and Vance, 1976; Mims et al., 2000). Even though callose deposition appears to be an ubiquitous plant defense response to invading pathogens and has been studied for over 150 years (DeBary, 1863), the specific function of callose in this defense reaction has remained unclear. On the one hand, callosic papillae have been found at sites of successful pathogen penetration (Aist, 1976), which has raised the question of the extent to which callose deposits contribute to penetration resistance. Further studies with *Arabidopsis thaliana* challenged the proposed function of callose as a physical barrier and structural reinforcement to slow pathogen invasion (Stone and Clarke, 1992; Voigt and Somerville, 2009). On the other hand, the powdery mildew resistant4 (*pmr4*) mutant revealed an increased resistance to powdery mildew infection, although callose was not deposited in papillae due to disruption of the stress-induced callose synthase PMR4 (also known as GLUCAN SYNTHASE LIKE5) in this mutant (Jacobs et al., 2003; Nishimura et al., 2003). Moreover, the mildew resistance locus O2-conditioned penetration resistance in *Arabidopsis* was found to be independent of PMR4-dependent callose deposition (Consonni et al., 2010).

In contrast, previous studies showed an active role of callose in penetration resistance mainly for Gramineae and in incompatible interactions. In addition, we recently demonstrated the effectiveness of callose deposition also in the compatible interaction of *Arabidopsis* with the powdery mildew *Golovinomyces cichoracearum* (Gc). *PMR4*-overexpressing *Arabidopsis* lines were completely resistant to Gc based on elevated early callose deposition at sites of attempted fungal penetration and the formation of a three-dimensional polymer network with cellulose fibrils of the preexisting cell wall (Ellinger et al., 2013; Eggert et al., 2014). However, enzymatic activation of the callose synthase PMR4 did not occur until translocation of the enzyme to the site of attempted fungal penetration. In unchallenged *PMR4*-overexpressing lines, an additional callose synthase activity or callose deposition was not observed (Ellinger et al., 2013). For
both, activation and translocation of callose synthases, GTPases might be involved. In yeast (Saccharomyces cerevisiae), the activity of the callose synthase homologs FKS06 SENSITIVITY1 (FKS1) and FKS2 is regulated by the GTPase Rh1 (Qadota et al., 1996; Calonge et al., 2003). In Arabidopsis, the Rh-like GTPase Rop1 is required for callose biosynthesis at the cell plate. Here, Rop1 interacts with a UDP-glucose transferase, a putative subunit of callose synthase complex (Hong et al., 2001a). For the translocation of PMR4 to the fungal penetration site, our recent results suggest transport via vesicle-like bodies (Ellinger et al., 2013). In general, GTPases can play a role in the correct polarized delivery of cargo and enzymes to sites of new cell wall synthesis by regulating membrane identity and vesicle budding, uncoating, motility, and fusion of the vesicle with the target membrane through the recruitment of effector proteins, which are, e.g., sorting adaptors, tethering factors, kinases, phosphatases, and motors (Lycett, 2008; Stenmark, 2009). The interaction or activation of those multiple effectors is regulated by the GTPase regulatory cycle in which GTPases cycle between an active GTP-bound and an inactive GDP-bound state. In their active form, they are often membrane-associated and perform their interaction with effector proteins. The inactivation of the regulatory activity is stimulated by GTPase activating proteins in addition to the inherent GTPase activity (Stenmark, 2009).

Our study aimed to identify and characterize GTPases that are involved in stress-induced callose biosynthesis. Based on the screening of publicly available microarray databases, we identified the monomeric GTPase RabA4c, which is also named SMALL MOLECULAR WEIGHT G-PROTEIN1, as a putative candidate. Its expression is induced after callose-inducing stress, such as wounding, elicitor treatment, or pathogen infection. RabA4c belongs to the RabA GTPase subgroup, which consists of 26 members in Arabidopsis (Vernoud et al., 2003). In general, Rab GTPases can be involved in the regulation of polarized cargo delivery between the Golgi network and the plasma membrane at sites of new cell wall synthesis in the growing tip (Lycett, 2008; Nielsen et al., 2008). The localization of Rab GTPases in the trans-Golgi network was based on amino acid sequence similarities (Vernoud et al., 2003). An involvement of GTPases (Hong et al., 2001b) as well as the trans-Golgi network (Cai et al., 2011) has also been shown in those developmental processes that include callose deposition, e.g., at the cell plate, plasmodesmatal canals, root hairs, spiral thickenings in tracheids, and sieve plates of phloem elements as well as around pollen mother cells and in pollen tubes (Kauss et al., 1983; Stone and Clarke, 1992). RabA4c has four direct homologs that are partially characterized. While the function and localization of RabA4a is unknown and RabA4e is classified as a pseudogene by TAIR (http://www.arabidopsis.org/index.jsp), RabA4b and RabA4d are necessary for the polarized transport via a secretory pathway to the growing tip. The GTPase RabA4b localized in membrane compartments at the tip of growing root hair cells (Preuss et al., 2004) and RabA4d in the tip of growing pollen tubes where the disruption mutant rabA4d revealed an altered distribution of pectin but not callose (Szumlanski and Nielsen, 2009). For RabA4c, studies in Arabidopsis roots suggest a function of this Rab GTPase in membrane trafficking during recovery from chilling stress, where RabA4c promoter activity colocalized with the region of maximum superoxide and hydrogen peroxide accumulation (Einset et al., 2007).

To investigate a possible role of RabA4c in stress-induced callose biosynthesis, we generated transgenic Arabidopsis lines expressing RabA4c under the control of the constitutive cauliflower mosaic virus promoter 35S. These RabA4c overexpression lines revealed a complete penetration resistance to the adapted powdery mildew Gc due to early elevated callose production at sites of attempted fungal penetration. Because this disease phenotype resembled the phenotype recently observed in PMR4 overexpression lines (Ellinger et al., 2013), we anticipated a direct interaction between RabA4c and the callose synthase PMR4. Our data confirmed this assumption and indicate that RabA4c enhances early, PMR4-dependent callose biosynthesis in an interaction where PMR4 acts as an effector protein of RabA4c.

RESULTS

RabA4c Expression during Powdery Mildew Infection

In our study, we wanted to elucidate the regulation of stress-induced callose biosynthesis in Arabidopsis. Based on the results in yeast where small GTPases regulate the callose synthase FKS1 (Levin, 2005), we started to identify GTPase genes that show increased expression after biotic stress in publicly available Arabidopsis microarray data using the Genevestigator tool (http://www.genevestigator.ethz.ch; Zimmermann et al., 2004; Hruz et al., 2008) and the e-FP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Schmid et al., 2005). One of the candidate GTPase genes was RabA4c, which was induced by various fungi as well as by application of the callose-inducing fungal elicitor chitin or its derivate chitosan (Iriti and Faoro, 2009) and the bacterial elicitor peptide flg22 (Gómez-Gómez et al., 1999) (Supplemental Figure 1).

To verify the microarray data showing an induced transcription of RabA4c after biotic stress, we performed quantitative real-time PCR (qPCR) analysis of 3-week-old Arabidopsis leaf tissue. We focused on early stages of Gc infection because callose deposition in leaves already occurs 6 h postinoculation (hpi) (Ellinger et al., 2013). Hence, a putative induction of callose synthase activity by GTPases, e.g., RabA4c, might even start before a visible callose deposit is formed. At 3 hpi with Gc, the RabA4c expression level was almost 6 times higher than before inoculation. Subsequently, RabA4c expression decreased to the level in untreated leaves and did not change during later stages of infection at 12 and 24 hpi (Figure 1).

Disease Phenotypes of Arabidopsis RabA4c Disruption and Overexpression Lines

For further analysis of a putative role of RabA4c in pathogen-induced callose formation, we examined the RabA4c T-DNA line (rabA4c-1) that was previously confirmed as a RabA4c disruption by Einset et al. (2007) and a second allele of an independent T-DNA line (rabA4c-2) that also resulted in a RabA4c disruption (Supplemental Figure 2). Both disruption lines did not show
a growth or developmental phenotype under standard cultivation conditions and were susceptible to the virulent powdery mildew Gc, which was indicated by the wild-type-like, whitish powdery appearance of leaves 7 d postinoculation (dpi) (Supplemental Figure 3A). Further characterization of the rabA4c disruption lines revealed that the strong Gc coverage of the leaves (Figure 2A) was associated with callose accumulation at fungal penetration sites (Figure 2B) and formation of a dense hyphal network with a fungal conidiation (Figure 2C) like on wild-type leaves (Figure 3). Despite the lack of a difference in disease phenotypes at later stages of infection, we determined a delayed activation of callose biosynthesis in the rabA4c disruption lines compared with the wild type. At 4 hpi, callose biosynthesis was already induced in the wild type, showing an almost 2 times higher callose synthase activity as in control leaves, whereas callose synthase activity in rabA4c-1 and rabA4c-2 leaves was not induced at this early time point of infection (Figure 2D). Callose synthase activity in rabA4c disruption mutants was not different from the wild type at later stages of infection apart from 24 hpi, where rabA4c-2 showed higher callose synthase activity than the wild type, but activity was not significantly higher than in rabA4c-1 (Figure 2D). The observed delay in the induction of callose synthase activity in rabA4c disruption lines at 4 hpi with Gc correlated with a reduced occurrence of the first callose deposit at 6 hpi. In contrast, callose deposition was not different at 24 hpi when callose synthase activity of the rabA4c disruption lines was similar or even higher than in the wild type (Supplemental Figure 3B). The almost 10% increase in penetration success of Gc in epidermal leaf cells of the rabA4c disruption compared with the wild type 24 hpi revealed a higher susceptibility of these disruption lines toward an attack by a biotrophic pathogen (Supplemental Figure 3B).

In contrast to the rabA4c disruption lines, the generated RabA4c overexpression lines revealed a complete penetration resistance to powdery mildew, which resembled the resistance phenotype of PMR4 overexpression lines (Ellinger et al., 2013). At 7 dpi with Gc, leaves of d35S:gRabA4c (expression cassette contains genomic RabA4c sequence with one intron and without tag) lines that expressed RabA4c under control of double 35S promoter were free from fungal growth and penetration, which resulted in the absence of stress-induced callose formation.

\[ \text{Figure 1. Relative Expression of RabA4c during Powdery Mildew Infection.} \]

Three-week-old wild-type plants were inoculated with the adapted powdery mildew Gc. All tests were conducted with rosette leaves. Relative expression of RabA4c was determined by quantitative RT-PCR. RNA was isolated from infected leaf tissue and used as template in cDNA generation. Gene expression at 0 hpi was used as reference and Actin2 expression for normalization. Values represent the mean of four biologically independent experiments, each done with two technical replicates. "P < 0.05 Tukey's test. Error bars represent SE, and n = 4.

\[ \text{Figure 2. Characterization of rabA4c Disruption Mutants after Gc Infection.} \]

(A) to (C) Disease phenotype and callose deposition of rabA4c-2 representative for disruption mutant rabA4c-1 and the wild type (Col-0). (A) Three-week-old rabA4c-2 disruption mutants were inoculated with the adapted powdery mildew Gc. Infection phenotype 7 dpi. (B) and (C) Localization of callose deposits stained with aniline blue (fluorescent spots) (B) and fungal hyphal network stained with calcofluor white (C) on the rosette leaf surface of rabA4c-2 disruption mutants 7 dpi. Micrographs taken by confocal laser scanning microscopy. Bars = 200 μm.

(D) Callose synthase activity of isolated rosette leaf membranes of the wild type (Col-0) and rabA4c disruption mutants 0, 4, 12, 24, and 72 hpi with Gc. Membrane fractions from unchallenged leaves served as control. Activity was determined in a fluorescence-based assay detecting produced callose via emission of callose-bound aniline blue. Values represent the mean of six biologically independent experiments. Letters a and b indicate groups with P < 0.05 by Tukey's test. Error bars represent SE, and n = 9 of 9 independent leaves. [See online article for color version of this figure.]
The complete resistance phenotype was confirmed in 35S:RabA4c-DsRed lines (Figure 3) that expressed RabA4c C-terminal fused to the red fluorescent protein DsRed to allow a possible in planta localization and under control of the single 35S promoter. To exclude a general effect of RabA GTPase overexpression on powdery mildew resistance, we generated d35S:gRabA4d lines, which overexpressed RabA4d, a member of the RabA4 family showing the highest homology to RabA4c (Vernoud et al., 2003). Unlike RabA4c overexpression, we did not observe resistance to the powdery mildew Gc in d35S: gRabA4d lines, and the leaves exhibited the same whitish powdery appearance as wild-type leaves at 7 dpi (Supplemental Figure 4).

To test whether an aberrant induction of plant defense signaling pathways would be involved in the observed powdery mildew resistance of RabA4c overexpression lines (Figure 3), we examined the expression of marker genes in qPCR assays. Like PMR4 overexpression lines and in contrast to the pmr4 mutant (Nishimura et al., 2003), a hyperactivation of the salicylic acid pathway did not occur in the 35S:RabA4c-DsRed line after Gc infection. The expression of the ISOCHORISMATE SYNTHASE1 (ICS1) gene, a marker for salicylic acid synthesis (Wildermuth et al., 2001), was not induced (Supplemental Figure 5A). In addition, expression of the jasmonate marker gene CORONATINE INSENSITIVE1 (COI1) (Katsir et al., 2008) was neither induced in the powdery mildew-resistant 35S:RabA4c-DsRed line nor in the wild type after Gc infection (Supplemental Figure 5B). Expression of the abscisic acid marker gene RESPONSIVE TO DESSICATION 29B (RD29B) (Yamaguchi-Shinozaki and Shinozaki, 1994) was induced in infected wild-type leaves but not in the resistant 35S:RabA4c-DsRed line (Supplemental Figure 5C). We also did not observe differences in the expression of the MEKK1 gene between the wild type and the 35S:RabA4c-DsRed line (Supplemental Figure 5D). MEKK1 encodes a mitogen-activated protein kinase kinase kinase, which is involved in abiotic stress signaling (Mizoguchi et al., 1996) and defense responses to pathogens (Asai et al., 2002) as well as reactive oxygen species homeostasis (Nakagami et al., 2006; Pitzschke et al., 2009).

To verify that the observed resistance phenotype was based on the GTPase activity of RabA4c, we generated transgenic lines that overexpressed a dominant negative (dn) isoform of RabA4c. Based on sequence similarities to known dominant negative GTPases, we identified a putative guanidine nucleotide binding site and introduced a single amino acid shift from serine to asparagine at residue 29 (S29N) to create RabA4c(dn). Dominant-negative GTPases are thought to lose the ability to bind GTP and bind GDP instead or are free from nucleotides (Yang, 2002).

Figure 3. Overexpression of Native RabA4c Confers Complete Powdery Mildew Infection. Rosette leaves of 3-week-old RabA4c overexpression lines and wild-type plants were inoculated with Gc. 35S:RabA4c-DsRed expressed RabA4c (cDNA fragment without intron) fused to DsRed under control of the single 35S promoter, d35S:gRabA4c the genomic RabA4c fragment (with intron and without fusion tag) under control of the double 35S promoter. RabA4c(dn) indicates the dominant negative S29N mutation in these constructs. Left column, macroscopic disease phenotype 7 dpi; middle column, micrographs of callose deposition (fluorescence by aniline blue staining); right column, micrographs of hyphal growth (fluorescence by calcofluor white staining). Micrographs taken by confocal laser scanning microscopy. Bars = 200 μm.
In analogy to the RabA4c overexpression lines, we generated additional lines that contained expression cassettes with RabA4c(dn) either under control of the single 35S promoter and with DsRed fusion [35S:RabA4c(dn)-DsRed] or the double 35S promoter without DsRed fusion [d35S:grRabA4c(dn)]. Despite the usage of a double 35S promoter for RabA4c overexpression in d35S:grRabA4c and d35S:grRabA4c(dn) lines, RabA4c expression levels were comparable in all RabA4c overexpression lines (Supplemental Figure 6).

All RabA4c(dn) overexpression lines revealed a disease phenotype that was not different from the wild type. At 7 dpi with Gc, the leaves had a whitish powdery appearance, which was associated with a dense hyphal network with good fungal conidiation on the leaf surface and callose accumulation at fungal penetration sites (Figure 3).

To confirm a lack of GTPase activity in RabA4c(dn) due to the S29N mutation, we heterologously expressed RabA4c and RabA4c(dn) in Pichia pastoris. RabA4c and RabA4c(dn) were fused to the green fluorescent protein (GFP) variant mCitrine (mCit) (Griesbeck et al., 2001) to enable purification of the proteins by immune precipitation using GFP-Trap agarose beads (Supplemental Figure 7). Purified RabA4c-mCit showed GTPase activity, whereas purified RabA4c(dn)-mCit revealed only a residual activity that was not different from the empty vector control (Figure 4).

Callose Deposition during Early Time Points of Powdery Mildew Infection

For detailed analysis of callose formation that is associated with the observed complete powdery mildew resistance, we harvested wild-type, 35S:RabA4c-DsRed, and 35S:RabA4c(dn)-DsRed leaves 3, 6, 12, and 24 hpi with Gc. The leaves were stained with aniline blue (Ellinger et al., 2013) to examine callose deposition by confocal laser scanning microscopy. At 3 hpi, we observed a dot-like callose deposit at the appressorial germ tube of Gc only in epidermal cells of 35S:RabA4c-DsRed leaves (Figure 5). This callose deposit increased in size and revealed a pattern at 6 hpi, which was similar to that of the recently described PMR4 overexpression line (Ellinger et al., 2013), i.e., a core with a surrounding field of callose (Figure 5). At this time point, a dot-like callose deposit was first detectable at the appressorial germ tube of Gc in the susceptible 35S:RabA4c(dn)-DsRed line and in the wild type (Figure 5). Within the next 6 h of infection, Gc penetrated the cell wall and formed haustoria, which are fungal feeding structures (Szabo and Bushnell, 2001), in wild-type and 35S:RabA4c(dn)-DsRed epidermal leaf cells. The penetration process was accompanied by the occurrence of a second callose deposit that formed a callose-encased penetration peg (Figure 5) as previously described for the wild type (Micali et al., 2008; Ellinger et al., 2013). By contrast, the resistant 35S:RabA4c-DsRed line formed a second strong callose deposit at this site of attempted fungal penetration 12 hpi that had the same callose deposition pattern as the first callose deposit (Figure 5). Gc failed to successfully penetrate the cell wall of 35S:RabA4c-DsRed epidermal leaf cells at either the first or second callose deposit. We could not detect penetration pegs or haustoria formation in this resistant line. At 24 hpi, additional callose was deposited at the second callose deposit of the wild-type and the 35S:RabA4c(dn)-DsRed line, however, without blocking the previously formed penetration peg. The first callose deposit started to or was already degraded at this time point. In addition, growing secondary hyphae (Figure 5) continuously penetrated the epidermal cells, indicated by numerous callose deposits at later time points of infection, and started to form the hyphal network that covered the leaf surface 7 dpi (Figure 3). In epidermal leaf cells of the resistant 35S:RabA4c-DsRed line, a third, strong callose deposit was often detectable at an additional site of attempted fungal penetration at the appressorial germ tube 24 hpi (Figure 5). Moreover, Gc was not able to initiate the growth of a secondary hyphae, which could be related to the collapse of conidia that we often observed on the surfaces of 35S:RabA4c-DsRed leaves at this time point.

Subcellular Localization of RabA4c

For subcellular localization of RabA4c, we had to generate new RabA4c overexpression lines to improve in planta imaging. The emitted fluorescence from RabA4c-DsRed fusion constructs in 35S:RabA4c-DsRed and 35S:RabA4c(dn)-DsRed lines was barely detectable in confocal laser scanning microscopy. The fluorescent protein mCitrine was chosen as fluorophore for the fusion with RabA4c because we already showed that it did not interfere with the GTPase activity of RabA4c (Figure 4). Both, RabA4c-mCit and RabA4c(dn)-mCit were clearly detectable in confocal laser scanning microscopy in Arabidopsis lines with a stable overexpression of the respective constructs 35S:RabA4c-mCit and 35S:RabA4c(dn)-mCit (Figure 6A). As we could previously show for 35S:RabA4c-DsRed, the Gc penetration resistance of line 35S:RabA4c-mCit (Supplemental Figure 8) correlated with elevated callose formation at sites of attempted fungal penetration, with the characteristic appearance of a third callose deposit (Figure 7), which we found to be a reliable marker.
for penetration resistance. We did not detect enhanced early callose deposition in 35S:RabA4c(dn)-mCit lines, which was similar to our observations in lines d35S:gRabA4c(dn) and 35S:RabA4c(dn)-DsRed (Figure 3).

Interestingly, RabA4c-mCit and RabA4c(dn)-mCit were predominantly localized at the plasma membrane (PM) in unchallenged leaves of 3-week-old plants and only occasionally in punctate structures at the PM periphery (Figure 6A; Supplemental Figure 9A). We distinguished between a possible localization of RabA4c-mCit at the PM, the tonoplast, or the thin layer of cytoplasm between the PM and the tonoplast, by staining leaf samples of 35S:RabA4c-mCit and 35S:RabA4c(dn)-mCit lines with FM4-64. This lipophilic dye is incorporated into the outer leaflet of the plasma membrane lipid bilayer but also into membranes of organelles depending on the used concentration and incubation time (Bolte et al., 2004). Localization at the PM was confirmed in colocalization studies with a calculated overlap coefficient between RabA4c-mCit/Rab4c(dn)-mCit and FM4-64 of above 0.9 (Figure 6C). Colocalization of RabA4c-mCit/Rab4c(dn)-mCit and FM4-64 also occurred in punctuated structures at the PM periphery (Figure 6A). The specificity of the predominant PM localization was confirmed in colocalization studies with the specific PM marker protein PMrk, a fusion protein of the PM aquaporin At-PIP2A (Becker, 2012) and the red fluorescence tag mCherry (Nelson et al., 2007). After transient expression of PMrk in leaves of 35S:RabA4c-mCit and 35S:RabA4c(dn)-mCit lines, we observed a direct overlap of the mCitrine signal from RabA4c and RabA4c(dn) fusion constructs with the mCherry signal from the PM marker protein PMrk (Supplemental Figure 9A), which was confirmed in colocalization studies, with a calculated overlap coefficient between RabA4c-mCit/Rab4c(dn)-mCit and PMrk of above 0.9 (Supplemental Figure 9B). Control experiments showed that transiently expressed PMrk also localized in the PM in the absence of RabA4c-mCit or RabA4c(dn)-mCit overexpression (Supplemental Figure 9C).

To test whether the localization of RabA4c-mCit and RabA4c(dn)-mCit at the PM was based on a localization preference of the fluorescence tag mCitrine, we examined the mCitrine signal in 35S:mCitrine control lines. Unlike RabA4c-mCit and RabA4c(dn)-mCit, single mCitrine did not colocalize with the PM stain FM4-64, as indicated by the relatively low overlap coefficient of only 0.52 (Figure 6C), and was localized in the cytoplasm (Figure 6A; Supplemental Figure 9D). Because it was known that C-terminal prenylation, which is the transfer of hydrophobic farnesyl or geranyl-geranyl moieties to cysteines, of most Rab GTPases is required for their membrane targeting (Calero et al., 2003), we wanted to test whether C-terminal tagging of RabA4c-mCit would affect the subcellular distribution of this GTPase. Therefore, we generated constructs for RabA4c and RabA4c(dn) expression with an N-terminal mCherry fusion [35S:mCherryRabA4c and 35S:mCherryRabA4c(dn)], Agrobacterium tumefaciens-mediated, transient expression of mCherryRabA4c and mCherryRabA4c(dn) in epidermal leaf cells revealed a predominant subcellular localization at the PM (Figure 6B; Supplemental Figure 9E), which was indicated by an overlap coefficient of 0.96 with the lipophilic membrane dye FM143 (Meckel et al., 2004) (Figure 6C). The examination of epidermal leaf cells without additional staining with membrane dyes showed a Rab4c distribution in punctate structures that was more frequent for N-terminal-tagged

Figure 5. Elevated Callose Deposition at Early Time Points of Powdery Mildew Infection in the RabA4c Overexpression Line.

Three-week-old overexpression lines 35S:RabA4c-DsRed and 35S:RabA4c(dn)-DsRed with the dominant-negative (dn) isoform of RabA4c and wild-type plants (Col-0) were inoculated with the adapted powdery mildew Gc. Micrographs show callose deposition (fluorescence by aniline blue staining) at sites of attempted fungal penetration at 3, 6, 12, and 24 hpi. Micrographs are 3D projections taken by confocal laser scanning microscopy. 1st Cd, first callose deposit; 2nd Cd, second callose deposit; 3rd Cd, third callose deposit; Gt, appressorial germ tube; Cp, chloroplast; Cw, plant cell wall; Ha, haustorium; Pp, penetration peg; Sh, secondary hyphae.
RabA4c than for C-terminal-tagged RabA4c (Supplemental Figures 9A and 9E) and seemed to be partially masked by the application of membrane dyes in parallel approaches (Figures 6A and 6B). The microscopically determined localization of both the C-terminal-tagged RabA4c-mCit and the N-terminal-tagged mCherry-RabA4c at the PM with their additional occurrence in putatively membrane-enclosed punctate structures was confirmed in protein gel blot analysis. RabA4c-mCit and mCherry-RabA4c proteins were extracted from 4-week-old leaves, separated into a soluble, cytosolic fraction and membrane protein-containing fraction by ultracentrifugation (Supplemental Figure 10A), and detected in protein gel blot using antibodies against their respective fluorophore. Both RabA4c-mCit and mCherry-RabA4c were only detected in the insoluble protein fraction where H+-ATPase served as a plasma membrane marker protein, but not in the soluble fraction, where UDP-glucose pyrophosphorylase (Martz et al., 2002) was used as a marker for cytosolic proteins (Supplemental Figure 10B).

Previous experiments have shown that C-terminal tagging of Rab GTPases can prevent C-terminal prenylation and membrane localization (Calero et al., 2003), which can result in an accumulation of the nonprenylated GTPase in the cytoplasm (Schultheiss et al., 2003). Because we did not detect RabA4c-mCit in the cytoplasm in spite of its C-terminal tagging, we hypothesized that RabA4c would not be directly associated with the PM but with a membrane-bound protein, which would not require a C-terminal prenylation. Based on our results of an enhanced early, pathogen-induced callose deposition due to RabA4c overexpression, we tested whether disruption of the pathogen-induced callose synthase PMR4 would break PM localization of RabA4c. After crossing of 35S:RabA4c-mCit with the disruption mutant pmr4, we observed RabA4c-mCit localization not only at the cell periphery but also in the cytosol, where the protein fluorescence signal did not colocalize with the membrane dye FM4-64 (Figure 6A) and resembled the localization pattern of single mCitrine (Supplemental Figures 9D and 9E).

![Figure 6. Subcellular Localization of RabA4c in Unchallenged Epidermal Leaf Cells.](image-url)

Micrographs were taken by confocal laser scanning microscopy of Arabidopsis rosette leaves.
Graphs were taken by confocal laser scanning microscopy. Yellow color was assigned to mCitrine-emitted fluorescence, red color to FM4-64 membrane stain, and blue color to aniline blue-stained callose. Visualization and localization of RabA4c-mCit, FM4-64-stained membranes, and callose deposition at the site of attempted fungal penetration 6 and 24 hpi in maximum intensity 3D reconstruction (left and two central columns). 3D surface rendering at the same site with the view from the cytosol of the epidermal leaf cell to the plasma membrane (right column). Size of bars as indicated.

1st deposition at the site of attempted fungal penetration 6 and 24 hpi in maximum intensity 3D reconstruction (left and two central columns). 3D surface rendering at the same site with the view from the cytosol of the epidermal leaf cell to the plasma membrane (right column). Size of bars as indicated. 1st Cd, first callose deposit; 2nd Cd, second callose deposit; 3rd Cd, third callose deposit; Gt, appressorial germ tube; St, stomata.

Figure 7. Subcellular Localization of RabA4c-mCitrine in Epidermal Leaf Cells after Gc Inoculation.

Three-week-old 35S:RabA4c-mCit lines were inoculated with the virulent powdery mildew Gc. All tests were conducted with rosette leaves. Micrographs were taken by confocal laser scanning microscopy. Yellow color was assigned to mCitrine-emitted fluorescence, red color to FM4-64 membrane stain, and blue color to aniline blue-stained callose. Visualization and localization of RabA4c-mCit, FM4-64-stained membranes, and callose deposition at the site of attempted fungal penetration 6 and 24 hpi in maximum intensity 3D reconstruction (left and two central columns). 3D surface rendering at the same site with the view from the cytosol of the epidermal leaf cell to the plasma membrane (right column). Size of bars as indicated. 1st Cd, first callose deposit; 2nd Cd, second callose deposit; 3rd Cd, third callose deposit; Gt, appressorial germ tube; St, stomata.

9F). Loss of significance for the overlap coefficient between RabA4c-mCit and FM4-64 (coefficient < 0.9; Figure 6C) and strong accumulation of RabA4c-mCit in the cytosolic, soluble protein fraction as determined in protein gel blot analysis in the cross pmr4x35S:RabA4c-mCit (Supplemental Figure 10B) confirmed a PMR4-dependent PM association of RabA4c.

A possible direct interaction of RabA4c with the callose synthase PMR4 was further indicated by the localization of RabA4c-mCit during early time points of Gc infection from 6 to 24 hpi when conidia successfully germinated and first callose deposits were visible at sites of attempted fungal penetration (Figure 5). Aniline blue staining was used to visualize deposited callose and FM4-64 to visualize the PM and possible vesicle-like structures (Bolte et al., 2004). At 6 hpi with Gc, RabA4c-mCit was no longer detectable at the PM but colocalized with deposited callose underneath the site of attempted fungal penetration. At this early stage of infection, RabA4c-mCit revealed an unstructured, cloud-like localization, which changed to a more compact and structured localization at later stages of infection when RabA4c-mCit directly surrounded the callose deposit (Figure 7). At 24 hpi, RabA4c-mCit accumulated at newly formed, third callose deposits in the same way as previously observed at the first callose deposit at 6 hpi and at second callose deposit at 12 hpi. At the same time, RabA4c-mCit was no longer detectable at first callose deposits where callose started to degrade (Figure 7). Degradation of callose continued at the additional deposits, which resulted in an almost complete disappearance of callose deposits 48 hpi. This also explained the absence of callose plugs in penetration-resistant lines at 7 dpi compared with the wild type, where continuous penetration and haustoria formation induced strong callose formation (Figure 3). The observed degradation of callose deposits in the resistant 35S:RabA4c-mCit line correlated with the reappearance of RabA4c-mCit at the plasma membrane of attacked epidermal cells. The mCitrine signal in the control line 35S:mCitrine colocalized neither with the plasma membrane stain FM4-64 nor with callose deposits (Supplemental Figure 11). In summary, RabA4c-mCit accumulated and colocalized with developing callose deposits and resisted at these sites of attempted fungal penetration as long as callose did not start to degrade.

RabA4c as a Regulator of PMR4-Dependent Callose Biosynthesis

Combining colocalization of RabA4c with callose and strong callose accumulation in RabA4c overexpression lines at sites of attempted fungal penetration with PMR4-dependent PM localization of RabA4c, we hypothesized that this Rab GTPase may actively regulate stress-induced callose biosynthesis through direct interaction with PMR4. Studies in Arabidopsis where a GTPase functioned as a regulator of cell plate-specific callose biosynthesis (Hong et al., 2001b) supported this assumption.

To further study a possible interdependency of RabA4c and the stress-induced callose synthase PMR4, we monitored the disease phenotype in RabA4c overexpression lines crossed with the pmr4 disruption mutant after Gc infection. The homozygous pmr4x35S:RabA4c-mCit and pmr4x35S:RabA4c-DsRed lines showed a disease phenotype that was not different from the described phenotype of the single pmr4 mutant 7 dpi with Gc (Nishimura et al., 2003). Like the pmr4 mutant, leaves of pmr4x35S:RabA4c-mCit and pmr4x35S:RabA4c-DsRed turned yellow and became necrotic without macroscopically recognizable pathogen growth (Figure 8A; Supplemental Figure 12). Further microscopic analysis of the pmr4x35S:RabA4c-DsRed line revealed that the density of the hyphal network was strongly reduced compared with the wild type without the strong callose deposition that is characteristic for wild-type leaves after Gc infection (Figure 8B). Additionally, we examined the penetration success of Gc by counting the formation of haustoria in epidermal leaf cells 24 hpi. Penetration success was not different between the pmr4x35S:RabA4c-DsRed line and the pmr4 mutant, reaching rates of ~80% (Figure 8C). These rates were
significantly lower than in the wild type, with a penetration success rate of ~90%, but dramatically higher than in the 35S:RabA4c-DsRed, with almost no Gc penetration (Figure 8C), which resembled the penetration resistance of the PMR4 overexpression line 35S:PMR4-GFP (Ellinger et al., 2013). The disease phenotype and the penetration success rate of the crossed pmr4x35S:RabA4c-DsRed line did not differ from homozygous lines that we generated by transformation of the pmr4 mutant with the binary vector carrying the 35S:RabA4c-DsRed construct. These lines were additionally screened for callose formation after wounding. Like the pmr4 mutant, the 35S:RabA4c-DsRed/pm4 lines did not deposit callose at wounding sites, whereas wound-induced callose deposition occurred in both 35S:RabA4c-DsRed and the wild type, but was not different (Supplemental Figure 13).

Because RabA4c overexpression lines resembled the complete penetration resistance phenotype of the PMR4 overexpression line (Ellinger et al., 2013), and the complete penetration resistance phenotype of RabA4c overexpression lines depended on the presence of the callose synthase PMR4, as shown with the crossed pmr4x35S:RabA4c-DsRed line (Figure 8), we tested whether RabA4c would act as an inducer of PMR4 transcription. A further indication for a putative transcriptional regulation of a callose synthase gene was given by studies in yeast, where the GTPase Rho1 activated the callose synthase FKS1 via induction of FKS1 transcription (Qadota et al., 1996; Park and Bi, 2007). Therefore, we determined PMR4 expression in qPCR experiments during Gc infection. Within the first 24 hpi, the 35S:RabA4c-DsRed line did not show a significant induction of PMR4 transcription that would correlate with the observed enhanced early callose formation at sites of attempted fungal penetration (Figure 5). PMR4 expression was also not induced in the wild type or the 35S:RabA4c(dn)-DsRed line (Supplemental Figure 14). The gene expression results further supported our assumption of a direct interaction of RabA4c and PMR4 in PMR4-dependent callose biosynthesis rather than transcriptional regulation.

To further verify the indicated interaction of RabA4c with the callose synthase PMR4, we performed yeast two-hybrid assays where we used the hydrophilic, intracellular loop of PMR4 (PMR-IL) (Supplemental Figure 15A) as prey and RabA4c as well as RabA4c(dn) as bait. We detected a strong interaction of PMR4-IL with native RabA4c, whereas RabA4c(dn) did not interact with

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Rosette leaves of 3-week-old wild-type (Col-0), RabA4c, 35S:RabA4c-DsRed, pmr4 mutant, and the cross pmr4x35S:RabA4c-DsRed were inoculated with Gc.

(A) Disease phenotype at 7 dpi. pmr4x35S:RabA4c-DsRed resembled phenotype of the pmr4 mutant with yellow and necrotic leaves.

(B) Micrographs showing callose deposition and hyphal growth 7 dpi after callose staining with aniline blue. White squares indicate areas of magnification in right panels. Micrographs taken by confocal laser scanning microscopy. Size of bars as indicated.

(C) Quantification of penetration success determined by haustorium formation per conidium at 24 hpi. Values represent the mean of four independent biological experiments. Letters a, b, and c indicate groups with P < 0.05 by Tukey’s test. Error bars represent se, and n = 12.

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Figure 8. PMR4 Dependency of Induced Elevated Callose Deposition due to RabA4c Overexpression.
PMR4-IL. These results were reproducible in 3-amino-1,2,4-triazole tests that were used for initial screening and in subsequent β-galactosidase assays (Table 1; Supplemental Figure 15B). Further evidence for a bona fide interaction between RabA4c and PMR4 was provided by pull-down experiments with purified RabA4c-mCit from heterologous expression in P. pastoris (Figure 4) and PMR4-IL that we purified by a Strep-tag from Escherichia coli extracts. After coincubation of RabA4c and PMR4-IL in presence of GTP and Ca2+ ions (Him et al., 2001), we were able to elute RabA4c-mCit together with PMR4-IL from GFP-Trap columns, as confirmed in protein gel blot analysis (Supplemental Figure 15C).

Finally, we provided evidence for a direct protein–protein interaction of RabA4c and PMR4 in planta by acceptor photobleaching-based fluorescence resonance energy transfer (FRET). We generated the PMR4 overexpression lines 35S: PMR4-mCit and 35S:PMR4-mCherry with a C-terminal mCitrine and mCherry tag. These lines were the basis for in planta analysis with possible mCit/mCherry FRET pairs after transient expression of either mCherryRabA4c or RabA4c-mCit in epidermal leaf cells. While live-cell imaging confirmed colocalization of tagged RabA4c with PMR4 (Figure 9A), the FRET efficiency of ~8% for the pair PMR4-mCit/mCherryRabA4c and ~10% for the pair PMR4-mCherry/RabA4c-mCit indicated direct protein–protein interaction in planta (Figure 9B). FRET efficiencies of these FRET pairs were significantly higher than the FRET efficiency of the pair RabA4c-mCit/PMR4 (with mCherry tag). We chose this pair as a control because of the demonstrated colocalization of RabA4c-mCit and PMR4 in imaging analysis (Supplemental Figure 9B) but the unlikely protein–protein interaction. In addition, the positive FRET results of the PMR4/RabA4c pairs also revealed that in planta protein–protein interaction was also independent of the tagged terminus of RabA4c.

DISCUSSION

The linear (1,3)-β-glucan callose is the most common chemical constituent of plant defense-related cell wall thickenings, so-called papillae (Aspinall and Kessler, 1957), which are formed at sites of fungal penetration. Studies with Arabidopsis lines where a delayed (Assaad et al., 2004; Nielsen et al., 2012) or enhanced early callose deposition (Ellinger et al., 2013) was examined suggest that the correct timing of papillae formation and callose deposition is critical for powdery mildew resistance.

In this study, we generated and characterized Arabidopsis lines with an overexpression of the monomeric GTPase RabA4c that were completely resistant to the virulent powdery mildew Gc (Figure 3; Supplemental Figure 8). The penetration resistance was based on enhanced early callose deposition at sites of fungal penetration (Figure 5), which resembled the resistance phenotype of the recently described Arabidopsis line with an overexpression of the stress-induced callose synthase gene PMR4 (Ellinger et al., 2013). Expression analyses of marker genes (Supplemental Figure 5) excluded a putative hyperinduction of additional pathways involved in plant defense response, which would contribute to powdery mildew resistance. In general, our findings highlight the active role of callose in the establishment of penetration resistance to adapted powdery mildews. Elevated and enhanced callose biosynthesis is reported here as an alternative defense strategy to the mlo (MILDWE RESISTANCE LOCUS O)-based powdery mildew resistance that was shown to be independent of callose (Consonni et al., 2010). Even though we observed increased callose deposition after fungal inoculation, we did not find spontaneous callose deposition in unchallenged leaves of RabA4c overexpression lines as reported for the powdery mildew resistant Arabidopsis triple knockout mutant mlo2/6/12 (Consonni et al., 2006). Regarding callose deposition, the RabA4c overexpression lines strongly resembled the PMR4 overexpression line. Based on the strong analogy of the resistance phenotypes, we concluded that RabA4c could be involved in the regulation of PMR4-dependent callose biosynthesis in response to Gc infection. We excluded a RabA4c-dependent regulation of PMR4 expression based on qPCR results that did not show differences between RabA4c overexpression lines and the wild type (Supplemental Figure 14). Therefore, we hypothesized that RabA4c might directly interact with PMR4. This assumption was supported by the reported interaction of the GTPase Rop1 with the callose synthase GSL6 at forming cell plates during cytokinesis in Arabidopsis, where Rop1 might be a part of the predicted callose synthase complex (Hong et al., 2001b). The function of Rop1 might be the activation of GLS6 through interaction with the central hydrophilic loop to create a substrate channel to transfer UDP-glucose, the substrate of callose synthases (Hong et al., 2001b). We hypothesized that a similar mechanism might also apply to RabA4c/PMR4 interaction.

Table 1. Yeast Two-Hybrid Interactions

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Interact-Ion 3AT Assay</th>
<th>Activity β-Galactosidase Assay</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDEST22:PMR4-IL+</td>
<td>Strong</td>
<td>4.24, 7.87, 11.03</td>
<td>7.71*</td>
<td>1.96</td>
</tr>
<tr>
<td>pDEST22:PMR4-IL+</td>
<td>Negative</td>
<td>0.00, 0.00, 0.00</td>
<td>0.00</td>
<td>–</td>
</tr>
<tr>
<td>pDEST22:RaiGDS+</td>
<td>Strong</td>
<td>6.60, 6.10, 5.89</td>
<td>6.20*</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Intracellular loop of PMR4 (PMR4-IL) used as prey and RabA4c and RabA4c(dn) as bait. Three independent clones tested in each protein–protein interaction in the β-galactosidase assay. Relative activity values are shown for this assay. Positive control (strong protein–protein interaction): RaiGDS in the prey vector pDEST22 and Krev1 in the bait vector pDEST32.

*Activity values not significantly different. P > 0.05 in Tukey’s test.
The loss of enhanced early callose deposition and the associated complete penetration resistance to Gc in the crosses pmr4x35S:RabA4c-mCit and pmr4x35S:RabA4c-DsRed (Figure 8; Supplemental Figure 12) provided genetic evidence for a dependency on functional Rab4C for the resistance phenotype and the specificity for this callose synthase in RabA4c-dependent callose regulation during early stages of powdery mildew infection. Because we applied confocal laser scanning microscopy coupled with a highly sensitive gallium-arsenite-phosphate nondescanned photodetector for detection in this study, we found trace amounts of callose at fungal penetration sites 7 dpi with Gc in the cross and the pmr4 mutant (Figure 8), which we did not detect in our recent study with an epifluorescence microscope approach (Ellinger et al., 2013). These trace amounts of callose indicated that at least one additional callose synthase might be involved in pathogen-induced callose biosynthesis at later stages of infection. The assumption was supported by the slight increase in callose synthase activity that was measured in the pmr4 mutant 24 hpi with Gc (Ellinger et al., 2013) and the increase in callose formation in the pmr4 mutant after elicitor treatment (Luna et al., 2011). In this regard, we cannot rule out that RabA4c might interact with callose synthases other than PMR4. These putative interactions might already occur in untreated cells and would explain why membrane association of RabA4c was not completely lost in pmr4x35S:RabA4c-mCit lines, as shown in our protein fractionation experiments followed by protein gel blot analysis (Supplemental Figure 10B). However, in these putative interactions with callose synthases other than PMR4, RabA4c would not have the function to induce enhanced callose biosynthesis because we did not observe additional callose deposition in unchallenged leaves or after Gc infection in the crosses pmr4x35S:RabA4c-mCit and pmr4x35S:RabA4c-DsRed compared with pmr4.

In addition to indications from protein fractionation and genetic evidence for an interaction between RabA4c and the callose synthase PMR4, we provided biochemical and biophysical evidence for a direct protein-protein interaction. In yeast two-hybrid experiments, RabA4c, but not the inactive isoform RabA4c (dn) (Figure 4), interacted with the hydrophilic, intracellular loop of the callose synthase PMR4 (Table 1; Supplemental Figure 15B). This result correlated with enhanced early callose deposition and penetration resistance that we only detected in RabA4c overexpression lines but not in RabA4c(dn) overexpression lines (Figures 3 and 5; Supplemental Figure 8). Hence, the interaction between RabA4c and PMR4 and the associated enhanced callose production likely depended on the activity status of RabA4c, which indicated a mode of activation for RabA4c following the general description for GTPases (Yang, 2002). Additional pull-down assays confirmed the indicated direct interaction of RabA4c with the callose synthase PMR4 (Supplemental Figure 15C). In addition to the two biochemical methods, we also showed a direct protein-protein interaction between RabA4c and PMR4 in planta using the biophysical method of FRET efficiency determination (Figure 9).

Even though we were able to show that the activity status of RabA4c was essential for inducing enhanced callose deposition at early time points of Gc infection and penetration resistance in both tagged and nontagged RabA4c overexpression lines, it did not determine subcellular localization and translocation to infection sites because we did not find differences between RabA4c and RabA4c(dn) before or after Gc inoculation. Differences in localization of native and dominant-negative RabA4c might have been expected due to results from other Rab GTPases. Mutated RabA2, which was locked into either the GDP- or GTP-bound state, relocalized to the Golgi or plasma membrane in comparison to its native variant (Chow et al., 2008). Also, mutated RabE1d was not detected in intracellular punctate structures (i.e., Golgi) but was primarily found at the cell periphery (Speth et al., 2009).

In contrast, we could show that native and dominant-negative RabA4c were predominantly associated with the PM due to direct interaction with PM-bound callose synthase PMR4 (Ellinger et al., 2013), for which we provided genetic, biochemical, and biophysical evidence in this study.
However, we also observed RabA4c localizing in punctate structures (Figure 6; Supplemental Figure 9), which could be anticipated from localization to vesicle membranes of other RabA family members. These GTPases were shown to be involved in regulating vesicular trafficking between the plant trans-Golgi network and the PM (Vernoud et al., 2003) like RabA4b (Preuss et al., 2004; Chow et al., 2008; Szumlanski and Nielsen, 2009) and RabA4d (Preuss et al., 2004; Chow et al., 2008; Szumlanski and Nielsen, 2009). A similar localization was also shown for RabA4c (Choi et al., 2013); however, only after transient expression in Nicotiana benthamiana leaves. Nevertheless, our results suggest that direct interaction between RabA4c and PMR4 may be sufficient to recruit RabA4c to the PM localization, where these proteins appear to play a role in successful establishment of penetration resistance to the adapted powdery mildew Gc. A PMR4-dependent recruitment of RabA4c to the PM would also explain why this localization was not reported after transient expression in N. benthamiana leaves (Choi et al., 2013) where PMR4 is not present. Whereas proper geranylgeranylation would be important for membrane association and correct localization of RabA4c functioning in vesicular trafficking, as shown for other Rab GTPases (Calero et al., 2003; Schultheiss et al., 2003; Hálá et al., 2010) and indicated by its C-terminal CysCysXaaXaaXaa moiety with a high probability of geranylgeranylation transferase 2-mediated geranylgeranylation, it would not be required for RabA4c function in regulating callose biosynthesis through direct interaction with PMR4. This would also explain why putative alteration or disruption of geranylgeranylation in C-terminal tagged RabA4c neither altered recruitment of this GTPase to the PM compared with N-terminal tagged RabA4c, as shown in confocal laser scanning microscopy, protein gel blot, and FRET analysis, nor penetration resistance compared with overexpression of the untagged RabA4c (d35S: gRabA4c line). A further indicator for an influence of a disturbed or disrupted geranylgeranylation would have been an accumulation of RabA4c in the cytoplasm, which was known from cleavage experiments with Rac/Rop and Rab GTPases (Schultheiss et al., 2003; Hálá et al., 2010). Because we neither found C-terminal- nor N-terminal-tagged RabA4c in the cytosolic protein fraction in the presence of a functional callose synthase PMR4 (Figure 6; Supplemental Figure 10B), we confirmed that C-terminal tagging did not alter recruitment of RabA4c to the PM by PMR4. However, the decreased occurrence of C-terminal-tagged RabA4c in punctate structures in the vicinity of the PM compared with N-terminal tagged RabA4c may indicate an involvement of C-terminal geranylgeranylation for membrane association in possible vesicular trafficking.

Combining our results of a direct interaction of RabA4c with the hydrophilic loop of the callose synthase PMR4 with the observed delayed induction of callose synthase activity 4 hpi with Gc (Figure 2D) and the following reduced callose plug formation at infection sites 6 hpi (Supplemental Figure 3B) in the two independent disruption mutants rabA4c-1 and rabA4c-2, which could be the reason for the increased pathogen susceptibility (Supplemental Figure 3B), we concluded that RabA4c may act as an enhancer of pathogen-induced callose synthase activity at early stages of infection. Hence, PMR4 can be considered to be an effector protein of RabA4c. At later stages of infection, an enhancing effect of RabA4c on pathogen-induced callose synthase activity might not be required because we did not determine lower activity in rabA4c disruption mutants compared with the wild type (Figure 2D). The increased callose synthase activity in rabA4c disruption mutants at 24 hpi could be induced by the elevated penetration success of Gc (Supplemental Figure 3B) because callose deposition is a major defense response of Arabidopsis to fungal penetration.

A specific challenge in future experiments would be to visualize putative RabA4c transport processes in planta, which we expect to be directly connected to PMR4 translocation after powdery mildew infection.

**METHODS**

**Growth Conditions, Inoculations, and Cytology**

Arabidopsis thaliana wild-type (Columbia-0 [Col-0]), pmr4 (allele 1; Nishimura et al., 2003), rabA4c-1 (T-DNA line, SALK_002567, Nottingham Arabidopsis Stock Centre; Einsel et al., 2007), rabA4c-2 (T-DNA line, SALK_005306, Nottingham Arabidopsis Stock Centre), the RabA4c overexpression lines from this study 3SS:RabA4c-DeRed, 3SS:RabA4c(dn)-DsRed, 3SS:gRabA4c, 3SS:gRabA4c(dn), 3SS:RabA4c-mCherry, 3SS:RabA4c(dn)-mCherry, the PMR4 overexpression lines 3SS:PMR4-mCherry and 3SS:PMR4-mCherry, and control lines 3SS:mCherry, 3SS:gRabA4d, and PM marker line PMrkl (Nelson et al., 2007) as well as the powdery mildew Golovinomyces cichoracearum (strain UC51) were cultivated as described by Stein et al. (2006). Arabidopsis inoculations (3-week-old plants were used in all experiments) and aniline blue staining for cytological analyses followed the protocol of Stein et al. (2006). Calcofluor white (Sigma-Aldrich) staining to visualize the fungal structures followed manufacturer’s instructions. Wounding experiments were performed with a needle.

**Cloning and Plant Transformation**

To create the RabA4c fusion to DsRed under control of the 35S promoter, RabA4c (At5g47960) was amplified from Arabidopsis Col-0 cDNA; the 35S promoter was amplified from the pCAMBIA3300 vector (Cambia) and DsRed from the pDsRed-Express vector (Clontech). All fragments contained a 20-nucleotide overhang to the adjacent fragment for sequential fusion PCR. The resulting fusion construct, 35S:RabA4c-DsRed, was cloned into pCAMBIA3300 via ligatation at the EcoRI restriction site. For the generation of the expression cassettes d35S: gRabA4c and d3SS: mCherry, gRabA4c was amplified from Arabidopsis Col-0 genomic DNA (containing one intron) and mCitrine (mCit) from pUC-mCit vector (ShineGene) and cloned into the pENTR/D-TOPO vector (Life Technologies). Using Gateway cloning technology (Life Technologies), gRabA4c and mCit were cloned into the Gateway-compatible plant expression vector pCAM-d35S, containing a double 35S promoter upstream to the Gateway cassette cloned into the pCAMBIA3300 vector. The same cloning strategy as described for the line d3SS: gRabA4c was used to generate the overexpression line d3SS: gRabA4d, gRabA4d (At3g12160) was initially amplified from Arabidopsis Col-0 genomic DNA.

To create the RabA4c fusion to mCit under control of the double 35S promoter, RabA4c was amplified from the 3SS:RabA4c-DeRed expression cassette with primers containing attB-sites for subsequent cloning into the entry vector pDONR221 (Life Technologies). From this vector, RabA4c was cloned into the Gateway-compatible plant expression vector pU710 (DNA-Cloning Service), containing a Gateway cloning cassette with an upstream double 35S promoter and a downstream mCit tag to generate C-terminal mCit fusion proteins. To create the RabA4c fusion to mCherry under control of the double 35S promoter, we used the same
entry vector containing the RabA4c sequence as described above for generating the RabA4c fusion to mCitrine. From this vector, RabA4c was cloned into the Gateway-compatible plant expression vector p7U10 (DNA-Cloning Service), containing a Gateway cloning cassette with an upstream double 35S promoter and a downstream mCherry tag to generate N-terminal mCherry fusion constructs. The generation of the dominant negative (dn) RabA4c isoform due to the mutation of the serine residue 29 to asparagine (S29N) was facilitated by mismatch PCR of the respective codon of the RabA4c sequence (from TCT to AAT) in the generated plant expression vectors following the instructions of the QuikChange mutagenesis kit (Stratagene). To create the PMR4 fusion to mCitrine and mCherry, under control of the double 35S promoter, PMR4 (At4g03550) was amplified from the 3SS:PMR4-GFP expression cassette (Ellinger et al., 2013) with primers containing attB-sites for subsequent cloning into the entry vector pDONR221. From this vector, RabA4c was cloned into the Gateway-compatible plant expression vector p7U10 with downstream mCitrine and mCherry tag to generate C-terminal PMR4 fusion constructs. A kanamycin resistance cassette provides selection in bacteria in all generated expression vectors. Primer sequences for the cloning of the constructs are provided in Supplemental Table 1. The transformation of Arabidopsis with all generated plant expression vector and subsequent plant selection followed the description by Ellinger et al. (2013).

Expression Analysis and Callose Synthase Activity

Rosette leaves were collected from uninfected and Gc infected plants at 1, 3, 6, and 24 hpi. Each sample represented a pool of rosette leaves from three plants. Total RNA was extracted from leaves using the innuPREP plant RNA kit (Avecgene) following the manufacturer’s instructions. Three plants. Total RNA was extracted from leaves using the innuPREP plant RNA kit (Avecgene) following the manufacturer’s instructions. cDNA syntheses were performed with 0.5 μg of total RNA with Maxima Reverse Transcriptase Kit (Thermo Scientific) and diluted 1:20 prior to qPCR analysis. Determination of gene expression by qPCR was performed in a Light Cycler 480 (Roche) using Arabidopsis-specific TaqMan probes (Life Technologies) labeled with the fluorescent dye VIC for ACTIN2 (assay ID: At02335270_gH) for normalization and TaqMan probes labeled with FAM for RabA4c (assay ID: At02612996_g1), PMR4 (assay ID: At02209120_g1), GS1 (assay ID: At02286250_g1), COI1 (assay ID: At02324942_g1), RD29B (assay ID: At02320468_g1), and MEKK1 (assay ID: At02210336_g1) expression analysis.

Callose synthase activity was determined in membrane fractions of uninfected leaf tissue and 4, 12, 24, and 72 hpi with Gc as described by Voigt et al. (2006).

Transient Protein Expression in Rosette Leaves

Expression of RabA4c variants were done according to Kim et al. (2009) with slight variation: Before infiltration, 0.01% Triton X-100 was added to the infiltration media. Arabidopsis plants were grown in soil for 5 weeks under long-day conditions, and only one injection on each side of the leaf vein was performed. After infiltration, plants were incubated at room temperature in the dark for 12 h covered with a polyethylene film before they were put back into growth chambers with long-day conditions. Colocalization and FRET efficiency were analyzed on the fourth and fifth day after infiltration.

Confocal Laser Scanning Microscopy and Image Analysis

Leaf samples were mounted between a microscope slide and a cover slip in water. Z series were captured with the LSM 780 confocal laser scanning microscope (Carl Zeiss Microimaging) using a either a 10× objective (EC Plan-Neofluar 10×/0.30 M27) or a 63× objective (C-Apochromat 63×/1.20 W Korr M27). A complete list about the used excitation and emission wavelength is given in Supplemental Table 2. The 3D and maximum intensity projections as well as 3D surface rendering of Z series were generated with the ZEN 2010 operating software (Carl Zeiss Microimaging). For colocalization studies, scatterplot data were recorded from all structures seen in the micrograph. When calculating the overlap coefficient, all pixels having the same position in the mCitrine and FM4-64 channels were considered to be pairs. The intensity level of pixel P1 of every pair of pixels was interpreted as X coordinate and that of P2 as Y coordinate of the scatterplot. In case of an overlap, a diagonal line and an overlap coefficient near 1 is produced; otherwise, different localizations cause an irregular distribution in the scatterplot.

FRET Analysis (Acceptor Photobleaching Method)

All FRET measurements were captured with the LSM 780 confocal laser scanning microscope using the 40× water immersion objective (C-Apochromat 40×/1.20 W Korr M27) in channel mode. The FRET pairs consisted of mCitrine as donor and mCherry as acceptor using the setup for emission and excitation wavelength as indicated in Supplemental Table 2. Bleaching experiments were done as follows: Regions of interest (ROI) were chosen from those cells that revealed expression of both fluorescent proteins. Before starting the bleaching process, 10 pre-bleaching images were recorded in both channels using the ROI mean function of the Zeiss 2010 operating software. The average of these 10 intensities (I) from mCherry was used as I before bleaching. Afterwards, bleaching of mCherry in ROI (90 s with 561 nm at 50% of the acousto-optic tunable filter) was performed until mCherry was hardly detectable. Directly after bleaching, 5 to 10 images of the yellow and red channels were captured every 7.5 s. The average of the first five intensities after bleaching was used as I after bleaching, FRET efficiency (EF) was calculated as followed: EF = (I before bleaching − I after bleaching) / I before bleaching × 100%.

To evaluate considerable variations in EF within the data series, first Gaussian distribution was established using the Kolomorov-Smirnov test followed by significance analysis with ANOVA.

Yeast Two-Hybrid Studies

To analyze a putative interaction between the GTPase RabA4c and the hydrophilic, intracellular loop of callose synthase PMR4 (PMR4-IL: Supplemental Figure 15) in a yeast two-hybrid assay, bait and prey vectors provided by the ProQuest two-hybrid system (Life Technologies) were used. PMR4-IL was amplified from the expression cassette 3SS:PMR4-GFP of the respective plant transformation vector (Ellinger et al., 2013) with primers containing attB-sites for subsequent cloning into the entry vector pDONR221 and from there into the prey destination vector pDEST22 (Life Technologies) using Gateway cloning technology. The same cloning strategy was used to generate the Rab4c and Rab4c(dn) bait in the destination vector pDEST32 (Life Technologies) with a previous amplification of RabA4c and RabA4c(dn) from the 3SS:RabA4c-DsRed and 3SS:RabA4c(dn)-DsRed expression cassette, respectively. Primer sequences for the cloning of the constructs are provided in Supplemental Table 1. Subsequent steps in the yeast two-hybrid assay followed the manufacturer’s instructions of the ProQuest two-hybrid system. The 3-amino-1,2,4-triazole (3AT) assay was used for an initial screening of positive RabA4c-PMR4-IL interactions. Three independent colonies from the transformation plate were cultivated in 3 mL selection medium for 48 h. From these cultures, 1 μL was used to inoculate 200 μL selective dropout medium (-histidine, -leucine, -tryptophan, 100 mM 3AT) in a 96-well plate. The plate was incubated at 28°C under continuous shaking for 4 d. Cell growth was measured every 24 h in a plate reader at OD 600. To verify the interaction, β-galactosidase induction was quantified using o-nitrophenyl-β-D-galactopyranoside as a substrate as described in the ProQuest protocol. For both the 3AT and the β-galactosidase assay, we used yeast clones containing pEXP32/Krev1+pExp22/RalGDSwt as positive control and clones containing pEXP32/Krev1+pExp22/RalGDSm2 as negative control as recommended by the manufacturer’s protocol.
Heterologous RabA4c Expression in *Pichia pastoris* and Protein Purification

To create the RabA4c and RabA4c(dn) fusion to mCit for heterologous expression in *P. pastoris*, RabA4c and RabA4c(dn) were amplified from the 3SS:RabA4c-DsRed and 3SS:RabA4c(dn)-DsRed expression cassette, respectively, and mCit from the vector pUC-mCit. All fragments contained a 20-nucleotide overhang to the adjacent fragment for sequential fusion PCR. The resulting fusion constructs RabA4c-mCit and RabA4c(dn)-mCit were cloned into the expression vector pGAPZA (Invitrogen) via ligation at the EcoRI and KpnI restriction sites. The same cloning strategy was used to generate the control expression vector with GFP only. Primer sequences for the cloning of the constructs are provided in Supplemental Table 1. Transformation and cultivation of *P. pastoris* with zeocin selection followed the manual of pGAPZ vector (Invitrogen). RabA4c-mCit and RabA4c(dn)-mCit as well as GFP as control were purified by immunoprecipitation using the GFP-Trap (Chromotek) with a GFP binding protein coupled to agarose beads. Five hundred microliters of cell lysate from 100 mL *P. pastoris* cultures was incubated for 4 h at 4°C with GFP-Trap beads. Washing of the beads was done once with a 150 mM NaCl buffer and once with a 300 mM NaCl buffer. For protein gel blot analysis, proteins were eluted from the beads as recommended by the manufacturer.

Heterologous PMR4(IL)-Strep Expression in *Escherichia coli* and Protein Purification

To create the PMR4(IL)-Strep fusion construct for heterologous expression in *E. coli*, PMR4-IL was amplified from the expression construct 3SS:PMR4-GFP (Ellinger et al., 2013) using a forward primer with a BamHI linker and a reverse primer with a NcoI linker for subsequent cloning into the *E. coli* expression vector pASK-IBA7plus (IBA), containing an upstream Strep tag to generate N-terminal Strep fusion proteins. Procedures followed the manufacturer’s instructions. PMR4(IL)-Strep was heterologously expressed and purified from *E. coli* using the Strep-Tactin Superflow system (IBA) using 1 mL of gravity flow columns following the manufacturer’s instructions. Identity of purified PMR4(IL)-Strep was verified by liquid chromatography-tandem mass spectrometry analysis and protein gel blot hybridization using Strep-Tactin-HRP conjugate (IBA).

GTPase Activity Assay

GTPase activity of RabA4c-mCit and RabA4c(dn)-mCit was determined with a GTPase assay kit (Innova Biosciences) following the manufacturer's instructions. Due to denaturing conditions during elution for activity assay, the proteins were still bound to the beads of the GFP-Trap. For calculation of specific protein activities, protein amounts were determined after elution from the trap using the NanoOrange protein quantification kit (Invitrogen) following the manufacturer’s instructions.

Statistical Analysis

Descriptive statistics including the mean and se, along with the Tukey range test for multiple comparison procedure in conjunction with an ANOVA, were used to determine significant differences. P < 0.05 was considered significant.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL libraries under the following accession numbers: RabA4c (At5g47960) and PMR4 (At4g03550).

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Stress-Induced Expression of PMR4 and RabA4c Based on Microarray Analysis.

**Supplemental Figure 2.** Molecular Characterization of rabA4c Disruption Mutants.

**Supplemental Figure 3.** Disease Phenotype of rabA4c Disruption Mutants after Powdery Mildew Infection.

**Supplemental Figure 4.** Disease Phenotype of RabA4d Overexpression Lines after Powdery Mildew Infection.

**Supplemental Figure 5.** Relative Expression of Stress Hormone Marker Genes during Powdery Mildew Infection.

**Supplemental Figure 6.** Relative Expression of RabA4c in RabA4c Overexpression Arabidopsis Lines.

**Supplemental Figure 7.** Protein Purification of RabA4c-mCit with GFP-Trap.

**Supplemental Figure 8.** Disease Phenotype of 3SS:RabA4c-mCit after Powdery Mildew Infection.

**Supplemental Figure 9.** Subcellular Localization of C- and N-Terminal Tagged RabA4c.

**Supplemental Figure 10.** Subcellular Localization of RabA4c in Protein Extracts of Uninfected Leaves.

**Supplemental Figure 11.** Localization of mCit after Powdery Mildew Infection.

**Supplemental Figure 12.** Disease Phenotype of the Cross pmr4x3SS: RabA4c-mCit after Powdery Mildew Infection.

**Supplemental Figure 13.** Callose Deposition in Epidermal Leaf Cells after Wounding.

**Supplemental Figure 14.** Relative Expression of PMR4 during Powdery Mildew Infection.

**Supplemental Figure 15.** Protein-Protein Interaction between RabA4c and the Intracellular Loop of Callose Synthase PMR4 from Arabidopsis.

**Supplemental Table 1.** Sequences of Primers Used in Cloning.

**Supplemental Table 2.** Excitation and Emission Wavelengths Used in Confocal Laser Scanning Microscopy.

**Supplemental Methods.**

**Supplemental References.**

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