

Enhanced resistance in *Theobroma cacao* against oomycete and fungal pathogens by secretion of phosphatidylinositol-3-phosphate-binding proteins

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Keywords: *Theobroma cacao*, disease resistance, phosphatidylinositol-3-phosphate-binding protein, effectors, oomycetes, fungi.

Summary

The internalization of some oomycete and fungal pathogen effectors into host plant cells has been reported to be blocked by proteins that bind to the effectors' cell entry receptor, phosphatidylinositol-3-phosphate (PI3P). This finding suggested a novel strategy for disease control by engineering plants to secrete PI3P-binding proteins. In this study, we tested this strategy using the chocolate tree *Theobroma cacao*. Transient expression and secretion of four different PI3P-binding proteins in detached leaves of *T. cacao* greatly reduced infection by two oomycete pathogens, *Phytophthora tropicalis* and *Phytophthora palmivora*, which cause black pod disease. Lesion size and pathogen growth were reduced by up to 85%. Resistance was not conferred by proteins lacking a secretory leader, by proteins with mutations in their PI3P-binding site, or by a secreted PI4P-binding protein. Stably transformed, transgenic *T. cacao* plants expressing two different PI3P-binding proteins showed substantially enhanced resistance to both *P. tropicalis* and *P. palmivora*, as well as to the fungal pathogen *Colletotrichum theobromicola*. These results demonstrate that secretion of PI3P-binding proteins is an effective way to increase disease resistance in *T. cacao*, and potentially in other plants, against a broad spectrum of pathogens.

Introduction

Plant defence involves two overlapping tiers of responses (Jones and Dangl, 2006). The first is triggered when plants detect conserved microbial molecular signatures (microbe-associated molecular patterns—MAMPs), and is called PAMP-triggered immunity (PTI). PTI includes rapid production of reactive oxygen species, antimicrobial molecules such as phytoalexins, and pathogenesis-related (PR) proteins.

Successful pathogens have evolved effector proteins, which can inhibit host defence responses (Giraldo and Valent, 2013; Torto-Alalibo *et al.*, 2010; Tyler and Rouxel, 2013). For example, in the oomycete *Phytophthora sojae*, 22 of 49 effectors screened could suppress PAMP-triggered responses (Wang *et al.*, 2011). The second tier of plant defence involves detection of effector proteins by host resistance (R) proteins (usually but not always nucleotide-binding leucine-rich repeat proteins) and is termed effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI can be readily overcome if pathogen strains emerge that have lost expression of the effectors or carry variant effectors that are no longer recognized by the R

protein. Many effectors show high variability among pathogen species, so R-gene-mediated resistance is often ineffective against different species of a pathogen, even from the same genus (Giraldo and Valent, 2013; Tyler and Rouxel, 2013).

Given the uneven success of conventional R genes, other strategies targeting effectors, such as blocking their function, are of interest given the importance of effectors in establishment of disease. Effectors are typically delivered into the plant cell through either pathogen- or host-encoded machinery (Tyler *et al.*, 2013). Effector delivery may occur from apoplastic hyphae, from specialized intracellular hyphae, or from haustoria (Tyler *et al.*, 2013) which are specialized feeding structures developed from intracellular hyphae (Hahn and Mengden, 1997; Panstruga and Dodds, 2009).

Oomycete effectors carry a short conserved N-terminal motif, RXLR, followed by several acidic residues (dEER) (Jiang *et al.*, 2008) that are required for entry of the effectors into host cells (Dou *et al.*, 2008; Kale *et al.*, 2010; Tyler *et al.*, 2013; Whisson *et al.*, 2007). Some fungal pathogen effectors may gain entry into plant cells through the same or similar processes (Kale *et al.*, 2010; Plett *et al.*,

2011). The RxLR-dEER domain of oomycete effectors, and fungal effectors with RxLR-like motifs were found to bind to the lipid phosphatidylinositol-3-phosphate (PI3P) (Kale *et al.*, 2010; Plett *et al.*, 2011) which was demonstrated to be on the surface of plant and animal cells. PI3P is also necessary for endocytotic processes such as protein sorting and membrane trafficking (Corvera *et al.*, 1999; DeCamilli *et al.*, 1996; Kale *et al.*, 2010). By preventing effectors from binding PI3P using competing PI3P-binding proteins or inositol-1,3-diphosphate, it was demonstrated that binding of the effectors to PI3P was required for cell entry (Kale *et al.*, 2010; Plett *et al.*, 2011).

There are several classes of proteins that can recognize and bind to specific forms of phosphoinositides. Examples include pleckstrin homology (PH) domains, Phox homology (PX) domains, and Fab1, YOTB, Vac1 and EEA1 (FYVE) domains. Different PH domains can bind specifically to a diversity of phosphoinositides (Dowler *et al.*, 2000; Kutateladze, 2010; Lemmon, 2008). Phox homology (PX) domains usually bind to PI3P, and sometimes to PI4P (Lemmon, 2008). FYVE domains bind specifically to PI3P (Kutateladze, 2010). These PI-binding proteins play diverse roles in membrane trafficking, cell growth and signal transduction (Lemmon, 2008).

An example of a crop species in which no R-gene-mediated resistance has so far been found is *Theobroma cacao*, the chocolate tree. Diseases are a major cause of crop loss, reducing the cacao yield by an estimated 30% (about 810 000 tons) per year and causing much farmer hardship (Keane and Putter, 1992). One of the most damaging cacao diseases is black pod rot, which is caused predominantly by three *Phytophthora* species: *P. palmivora*, *P. megakarya* and *P. tropicalis* [cacao isolates of *P. tropicalis* were previously identified as *P. capsici* (Aragaki and Uchida, 2001), (Guest, 2007)]. These pathogens can attack all parts of the plant including leaves, but major losses result from infection of cacao pods resulting in necrosis, shrinkage and mummification. The genomes of these three pathogens encode numerous RxLR effectors (B. Tyler, B. Bailey, M. Guiltinan, unpublished data).

With the aim of developing a novel, broad-spectrum disease resistance technology for cacao and potentially for other crop plants, we targeted the cellular translocation of pathogen effector proteins mediated by PI3P. We engineered cacao plants to secrete PI3P-binding proteins with the aim of blocking effectors from binding PI3P and thus from entering plants to promote disease. We show here that both transient and stable expression of secreted PI3P-binding proteins in cacao substantially enhances resistance to *P. tropicalis* and *P. palmivora*, as well as to the fungal pathogen *Colletotrichum theobromicola*.

Results

Design and verification of PI3P-binding constructs

To test the efficacy of secreting PI3P-binding domains to confer disease resistance, a variety of domains representing different

classes of PI3P-binding proteins were chosen (Table 1). The PI3P-binding proteins chosen were the PH domain proteins PEPP1 (human) and GmPH1 (soya bean) (Dowler *et al.*, 2000), the PX domain protein VAM7p (yeast) (Lee *et al.*, 2006) and the Fab1, YOTB, Vac1 and EEA1 (FYVE) domain protein Hrs (mouse) (Kutateladze, 2006). A tandem repeat of the Hrs FYVE domain (Hrs-2x FYVE) was used to increase its binding affinity (Gillooly *et al.*, 2000). As a control, we included the human PH domain protein FAPP1 which binds to PI4P (DiNitto and Lambright, 2006; Dowler *et al.*, 2000; Lemmon, 2008). To target the PI-P-binding domains to the apoplastic space upon expression, the domains were fused to the secretory leader from the soya bean PR1a protein (Cutt *et al.*, 1988; van Esse *et al.*, 2006; Honee *et al.*, 1998). Further, to allow visualization of protein expression and localization, the PI-P-binding domains were fused with enhanced green fluorescent protein (EGFP) (Figure 1a).

Transient expression of PI-P-binding domains

To test the efficacy of the proteins in cacao leaves, we developed an *Agrobacterium*-mediated transient gene expression system (agro-infiltration) capable of expressing substantial amounts of the proteins in a large percentage of leaf cells (Shi *et al.*, 2013). The transgenes were driven by a very strong modified E12- Ω CaMV35S promoter (Mitsuhashi *et al.*, 1996). Two types of constructs were tested, those in which EGFP was directly fused to the test genes to create a fusion protein, and nonfusion constructs in which the two coding sequences were driven by separate promoters (Figure 1a). To quantify transcription of the transgenes after transient expression in the cacao leaves, quantitative reverse transcriptase-PCR (qRT-PCR) employing primer pairs separately spanning the PI-P-binding region and the EGFP region was used to measure transcript levels in leaves, 2 days after infiltration (Figure 2). Transcript levels following transient expression of each transgene were approximately the same in each case and similar to those of the endogenous controls *Theobroma cacao* *Acyl-Carrier Protein 1* (*TcACP1*) and *TcTubulin1*. When the expression of the two components of the fused transcripts was measured individually using the specific primer sets (PI-P-binding domain and EGFP), as expected, there were no significant differences. Similar results were also observed when the PI-P-binding domain genes and EGFP were driven by separate promoters.

Cacao leaves transiently expressing apoplast-targeted PI3P-binding domains show increased resistance to *P. tropicalis*

To test the effect of PI3P-binding domain expression on pathogen resistance, the PI3P-binding proteins were expressed in cacao leaves by agro-infiltration, and the expression was verified using the EGFP reporter protein (Figure 3a). The reduced level of green fluorescence observed with the fusion proteins relative to the

Table 1 The names, species of origin, accession numbers, class type, binding specificity and size of all the domains used in this study

Domain name	Species	Accession number	Domain type	Binding specificity	Length (kb)
VAM7p-PX	<i>Saccharomyces cerevisiae</i>	P32912.1	Phox homology (PX)	PI3P	0.4
Hrs-2x FYVE	<i>Mus musculus</i>	D50050	Fab1, YOTB, Vac1, EEA1 (FYVE)	PI3P	0.5
PEPP1-PH	<i>Homo sapiens</i>	AAG01896.1	Pleckstrin Homology (PH)	PI3P	0.45
GmPh1-PH	<i>Glycine max</i>	NP_001235232.1	Pleckstrin Homology (PH)	PI3P	0.45
FAPP1-PH	<i>Homo sapiens</i>	AAG15199.1	Pleckstrin Homology (PH)	PI4P	0.3

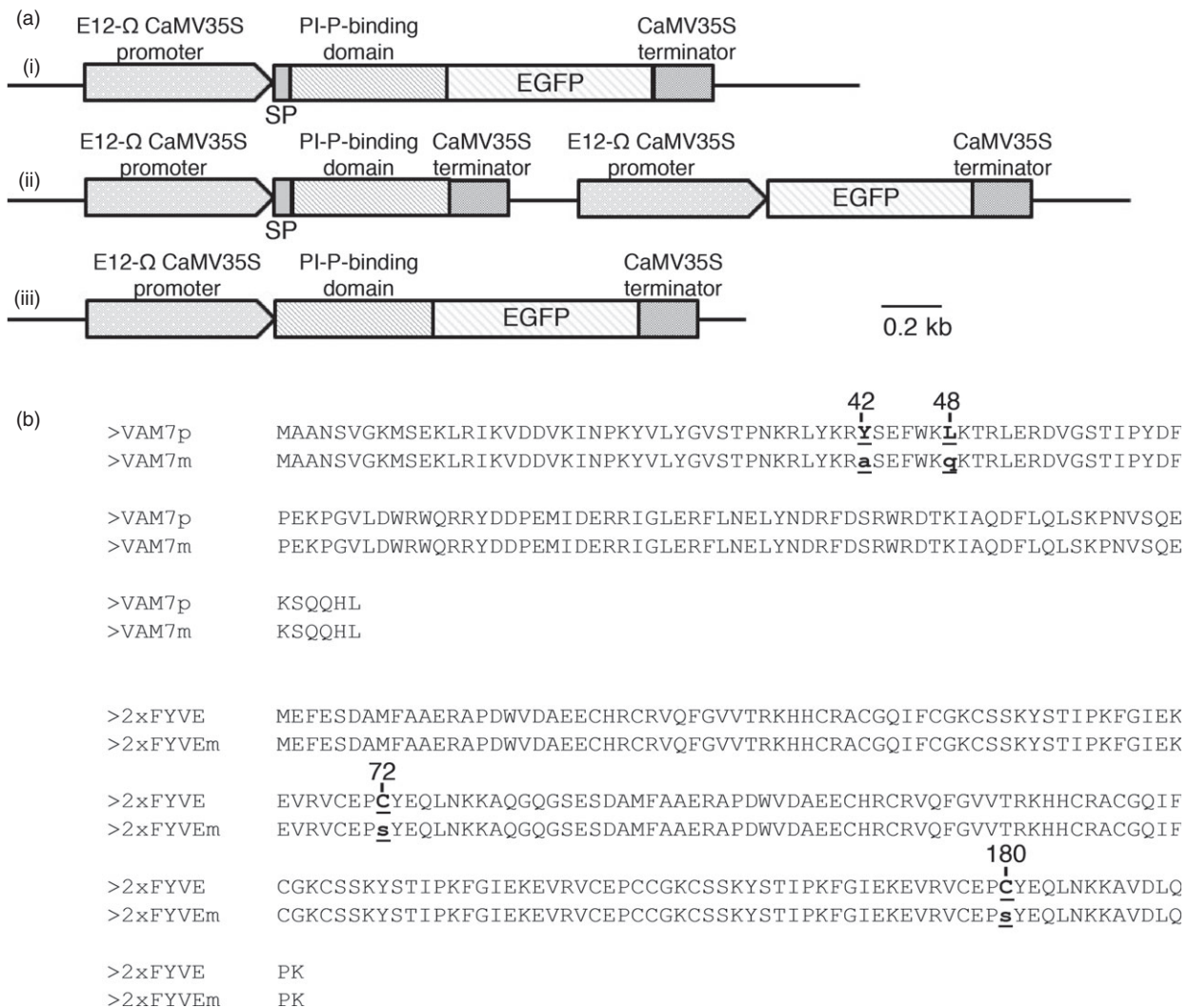


Figure 1 Design of constructs and mutant controls. (a) Structure of transgene cassettes used in this study. Constructs (i) and (ii) both include regions encoding a phosphoinositide (PI-P)-binding domain fused to a signal peptide (SP) under the control of a strong constitutive promoter (*35S_{E12}*). In (i), there is an EGFP reporter gene fused to the SP+PI-P construct (VAM7p, Hrs, PEPP1, GmPH1, FAPP1). In (ii), the EGFP is under the control of a separate *35S_{E12}* promoter (VAM7p, Hrs, PEPP1, VAM7 m). (iii) the PI-P-binding domain is fused to an EGFP reporter gene under the control of a *35S_{E12}* promoter, but without the signal peptide (PEPP1). (b) Amino acid sequences of two PI3P-binding domains and their mutated versions: VAM7p and its mutant, VAM7 m (Cheever *et al.*, 2000); and the duplicated FYVE domain from Hrs and its mutant, Hrsm (Kutateladze, 2006; Raiborg *et al.*, 2001).

EGFP-only control is likely a result of export of the fusion protein into the apoplastic space where the lower pH in the apoplastic space would quench the GFP signal. Weak GFP fluorescence observed in the cytoplasm in these cases is likely due to incomplete secretion of the proteins and/or re-entry of the secreted proteins into the cells (Kale *et al.*, 2010). After 48 h, agar plugs containing *P. tropicalis* isolate 73–74 were placed on each leaf (Figure 3b) alongside a control consisting of sterile agar plugs. Pathogen infection was evaluated after 3 days by two methods, lesion area (Figure 3c) and the relative amount of pathogen genomic DNA (Figure 3d). Pathogen DNA levels were measured by quantitative PCR (qPCR) using DNA isolated from standard-sized leaf discs, using primers specific for pathogen and cacao *Actin* genes. The ratio of these two measurements was used as an indicator of the relative amount of pathogen DNA present in each lesion, which is a proxy for the pathogen biomass.

As compared to the control transformation lacking a PI3P-binding protein, cacao leaves expressing the PI3P-binding domains from VAM7p, Hrs, PEPP1 and GmPH1 showed 55%–85% reduction both in the areas of the lesions and in *P. tropicalis* colonization (as measured by genomic DNA levels) (Figure 3). Inoculation experiments with cacao leaves expressing SP::VAM7p, SP::Hrs and SP::PEPP1 unfused to an EGFP protein showed no difference to results compared to leaves expressing fusion proteins (Figure S1). The expression of the PI4P-binding domain from FAPP1 resulted in lesion sizes that were not significantly different than the control leaves. Lastly, transformation of constructs encoding two different mutated domains, VAM7 m and Hrsm, that could no longer bind PI3P (Cheever *et al.*, 2001; Kutateladze, 2006; Raiborg *et al.*, 2001) (Figure 1b) had no significant effect on lesion size, nor on pathogen biomass, confirming that the PI3P-binding activity of these two proteins was required to produce resistance.

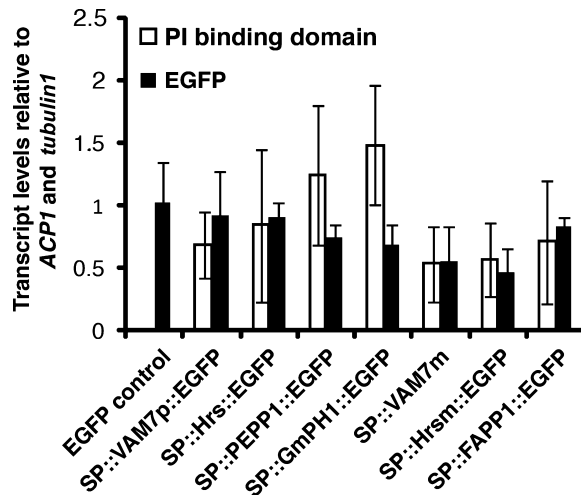


Figure 2 Measurement of the transcript levels from each construct following transient expression in detached cacao leaves using quantitative reverse transcriptase–PCR. Bars represent transcript levels as measured by primers for each respective PI-P-binding domain or for EGFP, standardized to the levels from internal control genes *TcACP1* and *TcTubulin1*. Measurements represent averages from three different leaves. Error bars represent standard errors. All levels show no statistically significant differences among each other.

Cacao leaves transiently expressing PI3P-binding domains show increased resistance to *P. palmivora*, a virulent agent of black pod rot

To test the efficacy of the constructs against a second cacao pathogen, leaves transiently expressing functional or mutated VAM7p domains or the EGFP-only control (Figure 4) were challenged with *P. palmivora*. Leaves expressing functional VAM7p showed significantly reduced lesion sizes and pathogen colonization as compared to the leaves expressing either mutated VAM7p domain or the EGFP-only control (Figure 4).

Apoplastic targeting of PI3P-binding domain proteins is required for resistance to *P. tropicalis*

The effector-blocking strategy assumes that the PI3P-binding domains must be targeted to the apoplastic space, to bind PI3P in the outer leaflet of the plasma membrane. To test this assumption, a PEPP1 PI3P-binding domain construct lacking a secretory leader was used (named PEPP1::EGFP in Figure 1a), which should result in protein accumulation solely in the cytoplasm. The expression of PEPP1::EGFP resulted in high levels of EGFP fluorescence in the cytoplasm approximately similar to the EGFP-only control, whereas, as expected, the SP::PEPP1::EGFP construct produced very little cytoplasmic EGFP (Figure 5a). Based on the lesion size and genomic qPCR bioassays, there was no significant difference in resistance conferred by the cytoplasmic-targeted PEPP1::EGFP compared with the control EGFP-only (Figure 5b). On the other hand, cacao leaves transformed with the apoplast-targeted SP::PEPP1::EGFP construct showed significantly smaller lesions and significantly less *P. tropicalis* colonization. These results demonstrate that to confer resistance to *P. tropicalis*, the PI3P-binding domains must be targeted to the apoplastic space.

Generation and verification of stable transgenic *T. cacao* plants expressing PI3P-binding domains from VAM7p, VAM7 mutant and Hrs

As a further test of the efficacy of the strategy, we generated stably transformed cacao plants expressing two of the PI3P-binding domains using *Agrobacterium*-mediated transformation (Maximova *et al.*, 2003). Stable transgenic plants carrying the SP::VAM7p construct (two independent lines), the SP::Hrs::EGFP construct (one line) and the SP::VAM7 m (non-PI3P-binding VAM7p mutant; one line) were produced and maintained in greenhouse conditions along with root cuttings of a control stable transgenic line carrying an EGFP-only construct.

The presence of the transgenes was confirmed using a PCR-based analysis (Maximova *et al.*, 2003) (Figure 6a). Three different sets of primers were designed to amplify a 100-bp sequence from the *TcActin* gene, a 500-bp sequence from the EGFP transgene and a 411-bp sequence (Bin19 backbone) located outside of the T-DNA region of the p126 transformation vector and not expected to be transferred to the transgenic plant genome. DNA was isolated from leaf tissues of five different genotypes: nontransformed cacao, EGFP-only transformants, SP::Hrs::EGFP transformants, SP::VAM7p transformants and SP::VAM7 m transformants. As expected, control p126A plasmid DNA produced amplified fragments with both Bin19 and EGFP primers. The nontransformed cacao samples produced amplified fragments only for cacao *actin*. Amplification of DNA from the transgenic cacao leaves using the three primer pairs resulted in products from *TcActin* and EGFP, but not from Bin19. The absence of Bin19 products from the stable transformants confirmed that there was no *Agrobacterium* present, nor any plasmid DNA contamination in the transgenic leaves. Therefore, it was inferred that genomic integration of the T-DNA had occurred in each of the transformants as expected, without inclusion of the flanking, non-T-DNA region of the Ti plasmids used.

The transcript levels of the constructs encoding the PI3P-binding domains and EGFP were measured using qRT-PCR (Figure 6b). The EGFP primers revealed transgene transcript levels about 90- to 180-fold higher than the endogenous *TcACP1* and *TcTubulin1* transcripts in the VAM7p-, Hrs- and VAM7 m-expressing lines and the control (EGFP-only). The Hrs primers revealed transgene transcript levels in the SP::Hrs::EGFP-expressing line about 300-fold higher than the *TcACP1* and *TcTubulin1* transcripts (the high signal from the Hrs primers relative to the EGFP primers likely results from the duplication of the FYVE domain). As expected, the Hrs primers did not amplify any sequences from the EGFP-only and SP::VAM7 lines. Likewise, the VAM7 primers revealed transgene transcripts around 200-fold higher than *TcACP1* and *TcTubulin1* in the two SP::VAM7p lines and 100-fold higher in the SP::VAM7 m line, but failed to amplify any sequences from the EGFP-only and SP::Hrs::EGFP lines.

To confirm that the secreted proteins remained intact in the apoplastic space, a Western blot was performed to detect Hrs fused to EGFP. Total protein was extracted from three leaves each from Scavina6, from the EGFP-only transformant and from the SP::Hrs::EGFP transformant and blotted with a GFP-specific antibody. The blot revealed no GFP-positive proteins in Scavina6, proteins of expected size (~32 kDa) for EGFP in the EGFP-only samples, and two protein bands in the SP::Hrs::EGFP samples: larger bands of about 50–52 kDa, which is the expected size of a Hrs::EGFP fusion protein, and 32 kDa, showing that some cleavage occurs between the Hrs and the EGFP proteins (Figure S2).

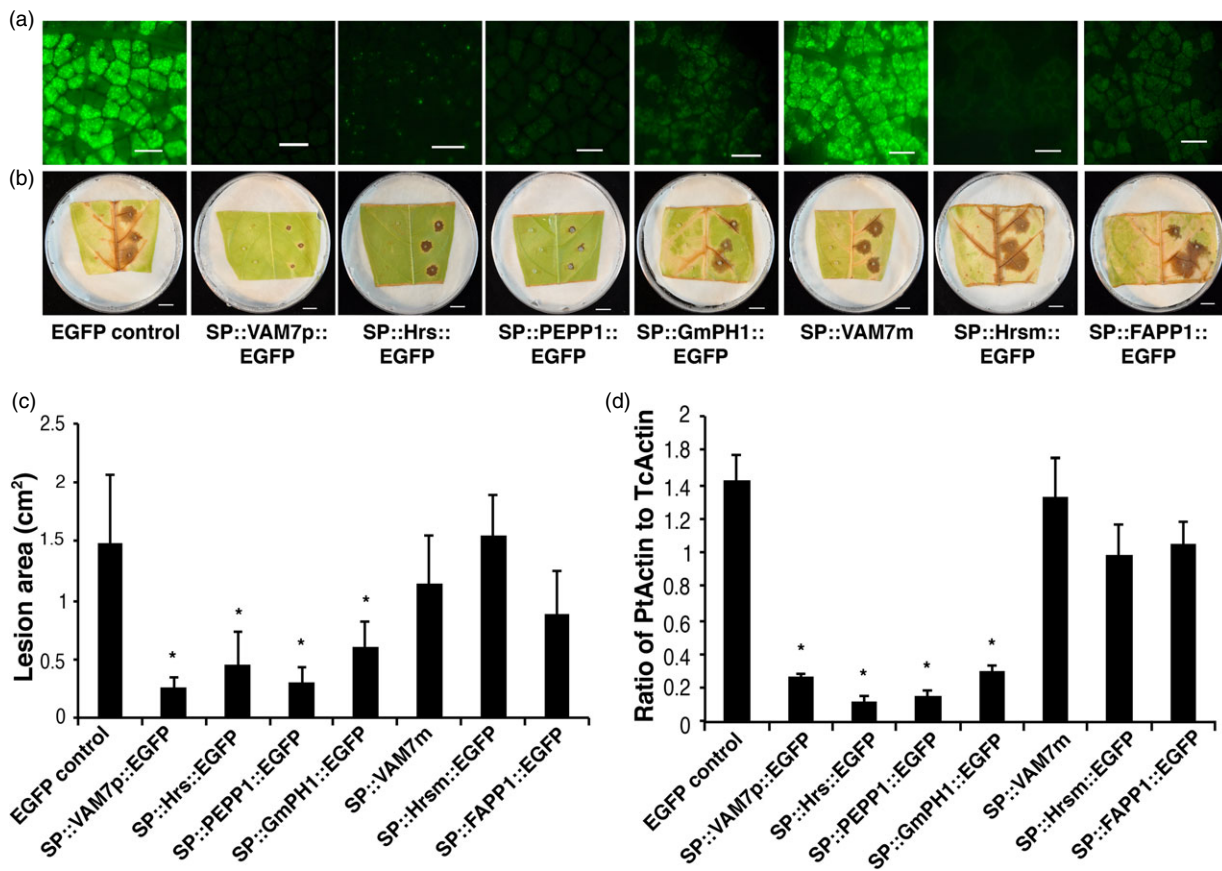


Figure 3 Transient expression of apoplast-targeted PI3P-binding domain proteins confers resistance to *Phytophthora tropicalis* in cacao. (a) Cacao leaf tissue transiently expressing EGFP 48 h after transformation by vacuum infiltration. Bars represent 250 μ m. (b) Representative transiently transformed leaves, 3 days postinoculation with 3-mm² agar plugs containing *P. tropicalis* mycelia (right) and clean agar plugs (left). Bars represent 1 cm. (c) Average lesion areas from each genotype 3 days postinoculation, calculated by ImageJ. Each bar represents the mean \pm standard error (SE) from three independent biological replicates, with 18 lesions per replicate. (d) Proliferation of *P. tropicalis* as determined by qPCR analysis of the genomic DNA ratio of *P. tropicalis* to *T. cacao* 3 days post-inoculation. Three lesions from each leaf piece were combined and extracted as a single genomic DNA sample. Each bar in the graph represents the mean \pm SE of three independent biological replicates, with each replicate consisting of three inoculation sites from two pieces from different leaves. In (c) and (d), asterisks indicate significant differences ($P < 0.05$) to the EGFP-only control as determined by ANOVA.

At a gross morphological and developmental level, all transgenic plants appeared phenotypically normal. No obvious visible differences in growth rate or morphology were observed in any of the plants relative to nontransgenic plants grown in identical conditions (Figure S3a). Visualization EGFP in leaves from stable transgenic plants expressing EGFP (cytoplasmic-localized EGFP) or SP::Hrs::EGFP (apoplastically-localized EGFP) (Figure S3b) resembled the distribution of EGFP in transient expression experiments (Figures 3 and 5); cytoplasmically localized EGFP appeared much brighter than apoplastically localized EGFP due to quenching. Visualization of EGFP in SP::VAM7p and SP::VAM7 m stable transgenic plants revealed a bright cytoplasmic EGFP signal, as the EGFP protein is targeted to the cytoplasm and is not produced as a fusion to the signal peptide and PI-binding protein (Figure S3b). To verify that the expression of the PI-binding proteins caused no severe membrane dysfunction or cell death, leaf discs from untransformed Scavina6, SP::Hrs::EGFP and SP::VAM7p-1 lines were stained with propidium iodide and compared with stained leaf discs that were previously killed with CuSO₄. In healthy tissue, it is expected that propidium iodide is excluded from the inside of the cells, showing only staining of the membrane. In dead tissue, the cell membrane is permeable, allowing for staining of cellular

contents. As shown in Figure S3c, Scavina6 leaf tissue killed with CuSO₄ showed staining of the cellular contents, but not of the membrane, whereas staining of Scavina6 leaves and leaves from the SP::Hrs::EGFP and SP::VAM7p transformants showed staining of cell membranes, with the stain being excluded from the intracellular space.

Transgenic *T. cacao* plants expressing apoplast-targeted PI3P-binding domains show enhanced resistance to two *Phytophthora* species

Leaves from stably transformed cacao plants expressing VAM7p, VAM7 m, Hrs and EGFP-only constructs were inoculated with 3-mm agar plugs containing mycelia of *P. tropicalis* isolate 73–74 or *P. palmivora* isolate DUK23.1. Leaves from a *T. cacao* transformant expressing an antimicrobial peptide, D4E1, previously shown to exhibit enhanced resistance to *P. palmivora* (Mejía *et al.*, 2012) were inoculated alongside as a positive control for resistance. Images of representative leaves 3 days after inoculation with the *Phytophthora* pathogens are shown in Figure 7a. Following inoculation with *P. tropicalis* 73–74, the *T. cacao* lines expressing Hrs showed a 69% reduction in lesion area, while the two independent VAM7p lines showed 74% and 79% reductions

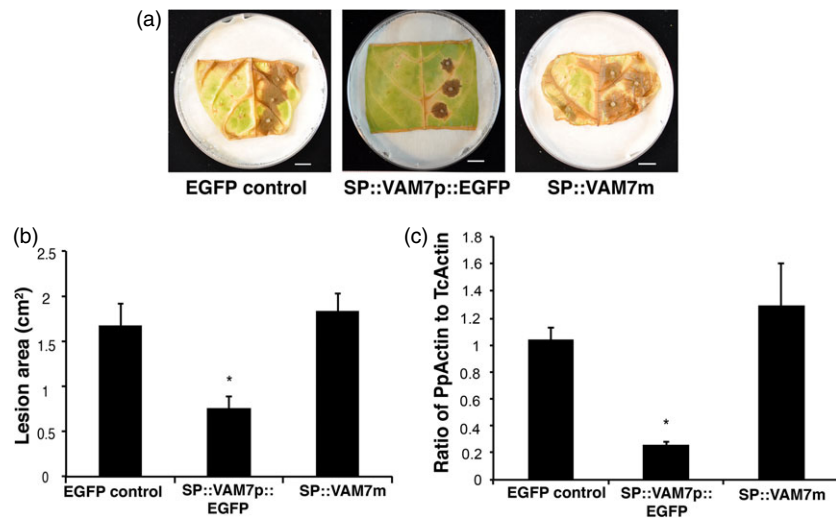


Figure 4 Transient expression of apoplast-targeted PI3P-binding domain VAM7p confers resistance to *Phytophthora palmivora*. (a) Representative transiently transformed leaves, 3 days postinoculation with agar plugs containing *P. palmivora* mycelium (right), and sterile agar plugs (left). Bars represent 1 cm. (b) Average lesion areas calculated with ImageJ, 3 days postinoculation by *P. palmivora*. Each bar represents the mean \pm SE of two independent biological replicates, with 18 lesions each. (c) Proliferation of *P. palmivora* as determined by qPCR analysis of the ratio of *P. palmivora* and *T. cacao* genomic DNAs 3 days postinoculation. Each bar in the graph represents the mean \pm SE of two independent replicates, with each replicate consisting three inoculation sites from two leaf pieces, each originating from different leaves. In (b) and (c), asterisks represent significant differences ($P < 0.05$) to the EGFP-only control as determined by ANOVA.

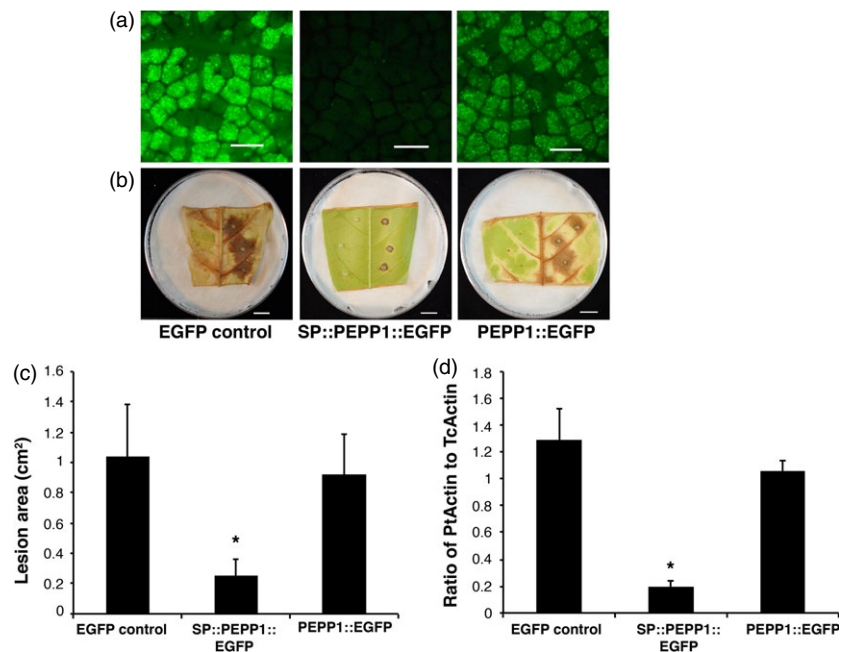


Figure 5 Resistance conferred by the PEPP1 PI3P-binding domain is dependent on targeting to the apoplast. (a) Cacao leaf tissue expressing EGFP, 48 h after transformation with each construct by vacuum infiltration. Bars represent 250 μ m. (b) Representative transiently transformed leaves expressing the indicated construct, 3 days postinoculation with agar plugs either containing *P. tropicalis* mycelium (left) or sterile water agar (right). Bars represent 1 cm. (c) Lesion areas calculated by ImageJ, 3 days postinoculation by *P. tropicalis*. Each bar represents the mean \pm SE from three independent biological replicates, with 18 lesions per replicate. (d) Proliferation of *P. tropicalis* as determined by qPCR analysis of the ratio of *P. tropicalis* to *T. cacao* genomic DNAs, 3 days postinoculation. Each bar in the graph represents the mean \pm SE of three independent replicates, with each replicate consisting of three inoculation sites from two pieces from different leaves. In (c) and (d), asterisks represent significant differences ($P < 0.05$) to the EGFP-only vector control as determined by ANOVA.

in lesion area. This was significantly lower compared to EGFP-only-expressing leaves, and slightly better than the 60% reduction in the positive control line expressing the peptide D4E1. The

mutant VAM7 m line showed about a 50% increase in lesion size that was not statistically significant, which shows that the PI3P-binding activity of the protein is required for resistance (Figure 7a,

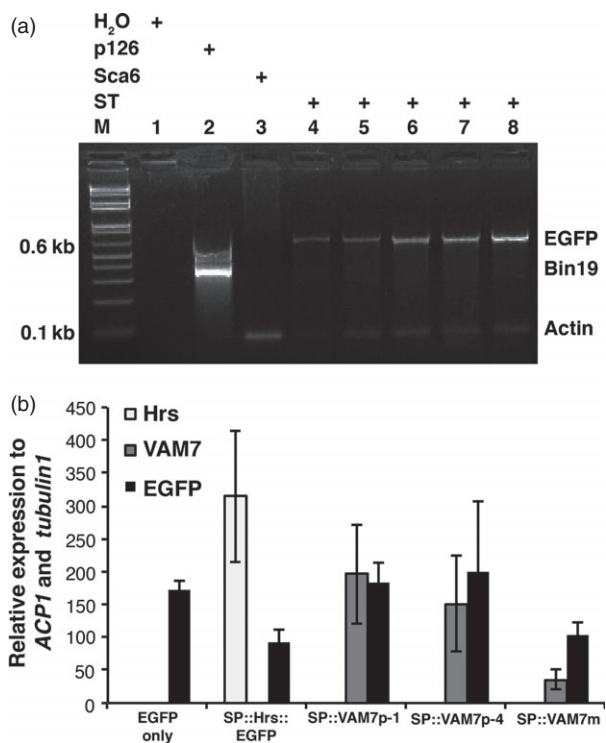


Figure 6 Verification of stable transformation of transgenic cacao lines with PI3P-binding protein constructs. (a) Test for transgene integration by genomic PCR analysis. Amplification of genomic DNA from nontransformed Scavina6 (Sca6, lane 3), stably transformed EGFP-only (lane 4), stably transformed SP::VAM7p::EGFP (line 1) (lane 5), stably transformed SP::VAM7p::EGFP (line 4) (lane 6), stably transformed SP::Hrs::EGFP (lane 7) and stably transformed SP::VAM7 m (lane 8) using a mixture of primers to amplify a 500-bp fragment from *EGFP*, a 100-bp fragment from endogenous *actin* and a 400-bp fragment from the p126 plasmid non-T-DNA backbone (Bin19). Positive control for Bin19 was plasmid DNA from p126 (lane 2). (b) Measurement of transgene transcript levels in leaves of cacao stable transformants SP::Hrs::EGFP, SP::VAM7p-1, SP::VAM7p-4, SP::VAM7 m and EGFP-only by qRT-PCR. Bars represent the average expression of each respective PI3P-binding domain-containing fusion gene, or *EGFP* relative to internal control genes *TcACP1* and *TcTubulin1* in three different leaves. Error bars indicate standard errors.

b). The reductions in lesion sizes in the D4E1-, Hrs- and VAM7p-expressing leaves were accompanied by 70%–90% reductions in *P. tropicalis* genomic DNA content, whereas a nonsignificant 40% reduction in *P. tropicalis* genomic DNA content was observed in the mutant VAM7 m line (Figure 7c). None of the differences in lesion sizes or genomic DNA content among the four resistant transformants were statistically significant. Following inoculation by the more virulent pathogen isolate *P. palmivora* DUK23.1, *T. cacao* leaves expressing Hrs and the two independent VAM7p lines showed lesion reductions of 59%, 48% and 60% respectively, compared to the EGFP-only transformant. The mutant VAM7 m lines showed a 25% increase in *P. palmivora* lesion size, which was not significantly different from the EGFP-only control (Figure 7a,b). These results were supported by the qPCR data, which showed an 83%, 93% and 81% reduction in the content of *P. palmivora* genomic DNA in the Hrs- and two VAM7p-expressing lines, and a nonsignificant 9% reduction in the nonbinding VAM7 m line (Figure 7c).

Stable transgenic *T. cacao* plants expressing functional PI3P-binding domains show strong resistance to the fungal pathogen *C. theobromicola*

To test the resistance of the transformants to a fungal pathogen, leaves from the Hrs-, VAM7p-, VAM7 m- and EGFP-only-expressing lines were inoculated with 10- μ L drops of a conidial suspension from two *C. theobromicola* isolates, 11–50 and 11–183. As for the *Phytophthora* resistance assays, leaves from the D4E1-expressing cacao line were used as a positive check for resistance (Mejía *et al.*, 2012). Images of representative leaves 4 days postinoculation by 11–50 and 11–183 are shown in Figure 8a. The three lines expressing the PI3P-binding domains from Hrs and VAM7p showed lesion areas after inoculation by *C. theobromicola* 11–50 that were reduced by 95%, 91% and 94%, respectively, compared to the EGFP-only control. The D4E1-expressing leaves showed a 75% reduction, which was significantly less resistance than that displayed by the PI3P-binding protein transformants. The mutant VAM7 m line showed about a 50% reduction in lesion size; however, this difference was not significant. When inoculated by a second *C. theobromicola* isolate, 11–183, the PI3P-binding protein transformants showed lesion areas reduced by 90%, 83% and 87%, respectively (Figure 8b), while the resistance conferred by the peptide was significantly weaker (55% reduction). The mutant VAM7 m line showed a 42% reduction in lesion area; however, this difference again was not significant. The strong resistance conferred by the PI3P-binding protein constructs was confirmed by qPCR quantification of *C. theobromicola* genomic DNA content: the genomic DNA content of 11–50 was reduced by 98% in the Hrs- and two VAM7p-expressing transgenic lines compared to the EGFP-only control line, while the expression of the peptide resulted in a 94% reduction of pathogen genomic DNA content. The genomic DNA content of 11–183 was reduced 97% in all three PI3P-binding lines and by 74% in the peptide-expressing line. The mutant VAM7 m line showed a 20% increase in both 11–50 and 11–183 genomic DNA (Figure 8c), showing that the slight decrease in lesion area was not associated with decreased pathogen colonization.

Discussion

Fungal and oomycete pathogens cause billions of dollars of economic loss each year throughout the world. For many crops, the lack of good resistance genes and difficult breeding systems means farmers must rely on chemical or cultural methods of disease management. Even when resistance genes are deployed in cultivars, the pathogen populations can often evolve rapidly to evade the resistance genes.

One critical step in the pathogen life cycle that is common across many bacterial, nematode, insect, fungal and oomycete species is the secretion and delivery of protein effectors into host cells, where they manipulate host physiology to promote the success of the pathogen (Torto-Alalibo *et al.*, 2010). The discovery that host cell entry by many oomycete effectors and several fungal effectors involves interactions with PI3P on the plasma membrane (Kale *et al.*, 2010; Plett *et al.*, 2011) suggested to us a possible target for blocking effector entry. We hypothesized that if sufficient amounts of PI3P-binding proteins could be expressed and targeted to the apoplast space, the proteins could competitively inhibit the PI3P binding and internalization of pathogen effectors, resulting in enhanced disease resistance.

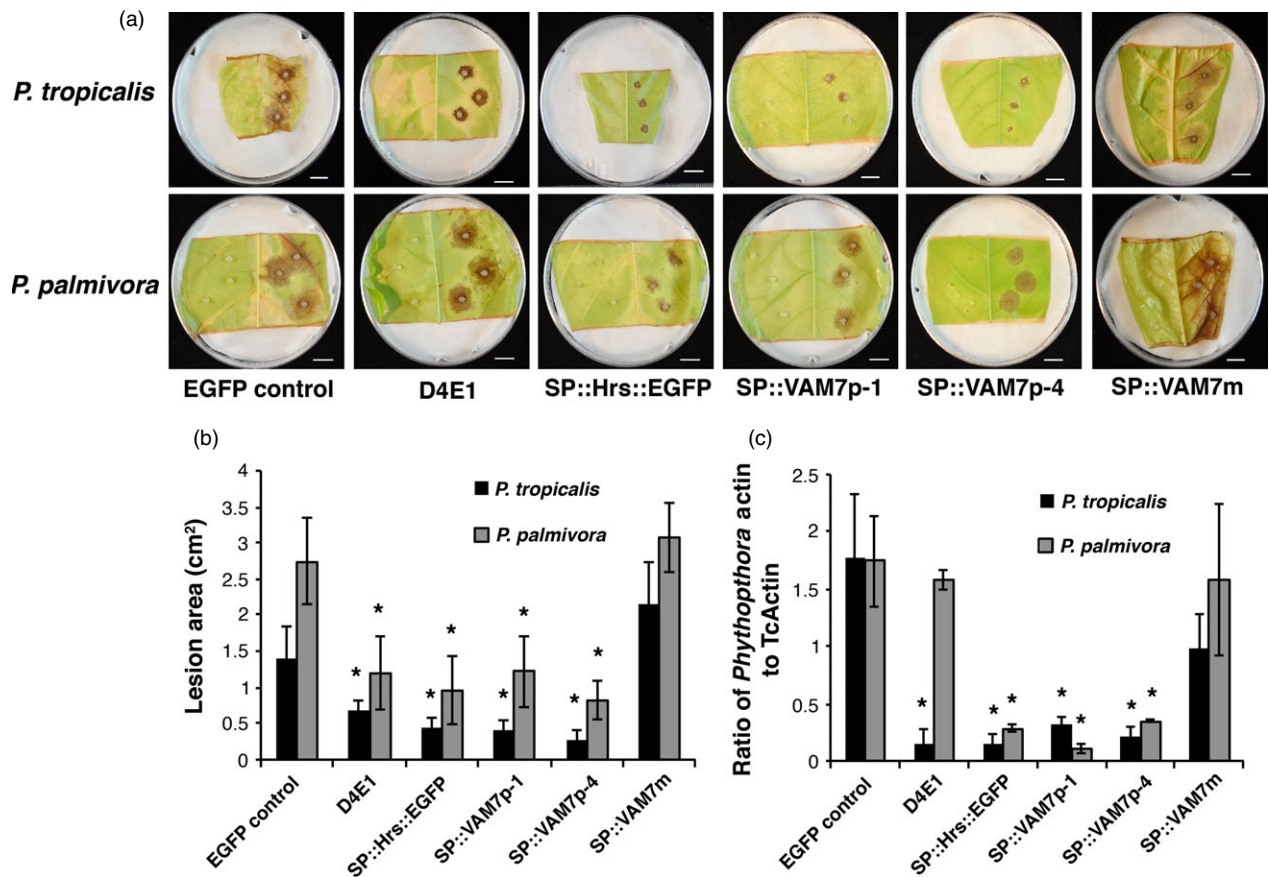


Figure 7 Expression of apoplast-targeted PI3P-binding domain proteins in cacao stable transformants confers resistance to *P. tropicalis* and *P. palmivora*. (a) Representative leaves from cacao stable transformants, 3 days postinoculation with *P. tropicalis* 73-74 (top row) or 3 days postinoculation with *P. palmivora* DUK23.1 (bottom row). Bars represent 1 cm. (b) Lesion area from each genotype calculated 3 days after inoculation with *P. tropicalis* or *P. palmivora*. Each bar represents the mean \pm SE from three independent biological replicates, with 18 lesions per replicate. (c) qPCR quantification of pathogen proliferation (3 days after inoculation in each case). Each bar in the graph represents the mean \pm SE of three independent replicates, with each replicate consisting of three inoculation sites from two pieces from different leaves. In (b) and (c), asterisks represent a significant difference ($P < 0.05$) to the EGFP-only control as determined by ANOVA.

Our results demonstrated that the expression of four completely different PI3P-binding proteins targeted to the apoplast space did provide resistance against two oomycete pathogens and a fungal pathogen. Three of the four proteins were previously shown to block entry by oomycete and fungal effectors (Kale *et al.*, 2010). No resistance was conferred by a PI4P-binding protein, nor by two proteins with mutations that abolish PI3P binding. Furthermore, targeting of the proteins to the apoplast was required to confer resistance. Resistance was observed both in transiently transformed cacao leaves and in three stably transformed whole plants. Together, the results support our original hypothesis that apoplastic expression of PI3P-binding proteins could be capable of reducing infection.

Although secretion of PI3P-binding proteins results in resistance, the precise mechanisms by which they confer resistance remain to be investigated. Although the blocking of effector entry is the most obvious mechanism, it is also possible that the proteins trigger some kind of resistance or priming response. The purpose of external PI3P on plant cell membranes is currently unknown, and a role in defence signalling is not ruled out currently. Our data also do not rule out that the site of action of the PI3P-binding proteins is within the endomembrane system rather than in the apoplast. The very strong resistance against *C. theobromicola*

suggests either that this pathogen utilizes PI3P to deliver its effectors into host cells (which is currently unknown) or that the plants are expressing a broader mechanism of resistance.

So far, we have produced three stable cacao transformants. The resistance phenotypes of these plants are similar and are consistent with the transient expression results. We are producing additional stable transformants, including plants expressing other PI3P-binding proteins, to further confirm the efficacy of these transgenes, and to improve the levels of resistance. So far, the transgenic plants have not been tested for resistance against the three most destructive pathogens of cacao, namely the oomycete *P. megakarya* and the fungi *Monilophthora perniciosa* (witches' broom) and *Monilophthora roreri* (frosty pod), because this will require maturation of whole plants and fruit production.

In summary, our data suggest that this technology offers a promising level of resistance against diverse pathogens that may be applicable to a wide range of crop species.

Experimental procedures

Binary vector construction

All sequences of PI-P-binding domains were obtained from public databases and synthesized by GenScript Corporation, with codon

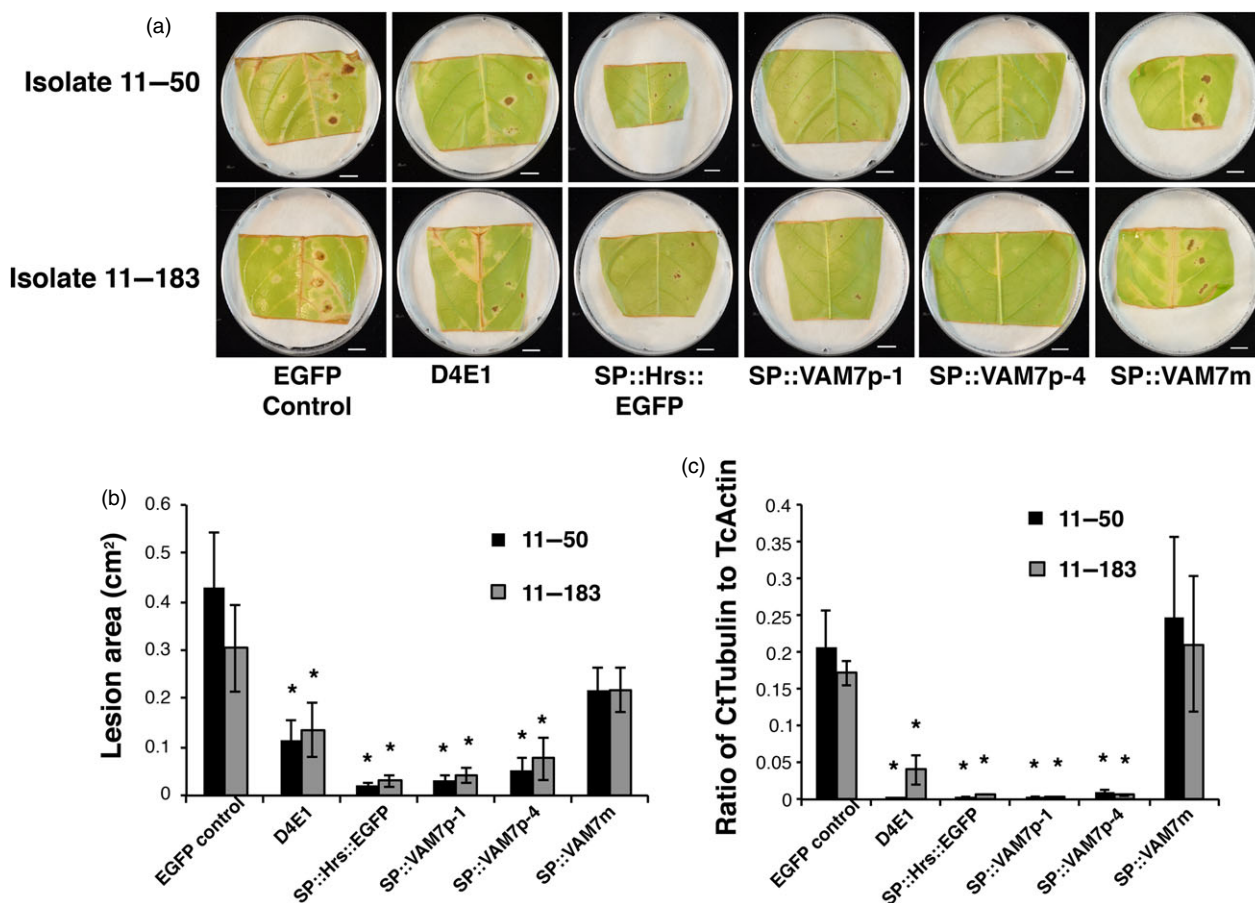


Figure 8 Expression of apoplast-targeted PI3P-binding domain proteins in cacao stable transformants confers resistance to two isolates of *Colletotrichum theobromicola*. (a) Representative leaves from cacao stable transformants 4 days after inoculation by *C. theobromicola* isolate 11-50 (top row) or *C. theobromicola* isolate 11-183 (bottom row). Bars represent 1 cm. (b) Lesion area of each *T. cacao* genotype measured 4 days after inoculation with *C. theobromicola* isolates 11-50 or 11-183. Bars represent the mean \pm SE of two independent biological replicates, with 18 lesions each. (c) qPCR quantification of *C. theobromicola* proliferation 4 days after inoculation. Each bar in the graph represents the mean \pm SE of two independent replicates, with each replicate consisting of three inoculation sites from two pieces from different leaves. In (b) and (c), asterisks represent a significant difference ($P < 0.05$) to the EGFP-only control as determined by ANOVA.

optimization for expression in *E. coli* and *N. tabacum*. These included human FAPP1-PH (AAG15199.1, residues 1–99), soya bean GmPH1 [NP_001235232.1, Glyma14g06560, residues 1–146; obtained by homology to AtPH1 (Dowler *et al.*, 2000)], human PEPP1-PH (AAG01896.1, residues 15–168), yeast VAM7p-PX (P32912.1, residues 1–134 with the substitution R73W) and mouse Hrs-2xFYVE [D50050, residues 147–223, as modified and duplicated by Gillooly *et al.* (2000)]. Site-specific mutagenesis of VAM7p-PX and Hrs-2xFYVE was performed using primer mutagenesis by PCR or by gene synthesis, respectively. Those mutations were previously described to abolish PI3P-binding by VAM7p-PX (Cheever *et al.*, 2001) or by Hrs (Kutateladze, 2006; Raiborg *et al.*, 2001). To create the EGFP-fused PI-P-binding domain vectors, binary vector pGH00-0126 (Maximova *et al.*, 2003) was made Gateway-compatible by adding the Gateway[®] cassette (Invitrogen, Waltham, MA) containing *attR* recombination sites flanking a *ccdB* gene and a chloramphenicol-resistance gene, generating vector 126gfp-gw. The PI-P-binding domains were cloned into 126gfp-gw, with (sp126gfp-gw) or without the signal peptide (SP) sequence from the *Glycine max* PR1a gene (NM_001251239, residues 1–27). Constructs where the PI-P-

binding domain was not fused to EGFP were created by adding *SpeI* and *HpaI* restriction sites onto the SP::PI-P-binding domain segment and subcloning into pGH00.0126 (GenBank: KF018690.1). All constructs were transformed into *Agrobacterium tumefaciens* strain AGL1 for both transient and stable transformations.

Transient and stable transformation of *T. cacao*

For transient transformation of detached cacao leaves, *A. tumefaciens* cells containing each transgene were grown as described in Maximova *et al.* (2003), induced with acetosyringone, and then vacuum-infiltrated into stage C leaves from cultivar Scavina6 as described in Shi *et al.* (2013). The Petri dishes were sealed and incubated at 25 °C for 2 days with light intensity of 145 m²/s and 14-h daylight. After 2 days, EGFP fluorescence was examined as described (Maximova *et al.*, 2003). Only leaves with green fluorescence coverage of 80% or more were further subjected to pathogen infection. Stable transformation of cacao was performed as described (Maximova *et al.*, 2003). Resulting stable transformants were grown for about 6 weeks in greenhouse conditions before leaves were sampled for further experiments.

Protein extraction and Western blot

Total protein was extracted from three leaves each of nontransformed Scavina6, stable transformed EGFP-only plants and stable transformed SP::Hrs::EGFP plants following the protocol of Pirovani *et al.* (2008). Western blotting was performed by electrophoresing 15 µg of total protein per sample on a 12% SDS-PAGE gel, followed by blotting onto a PVDF membrane (Millipore, Billerica, CA) using a wet transfer apparatus (BioRad, Hercules, CA). Post-transfer, the membrane was blocked with 2% bovine serum albumin in PBS-T (137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 1.8 mM KH₂PO₄; 0.1% Tween-20), rinsed and incubated for 16 h with rabbit anti-GFP primary antibody (Immunology Consultants Lab, Inc., Portland, OR) at 4 °C. The membrane was rinsed in PBS-T, incubated for 1 h at room temperature with 1 : 10 000 dilution of HRP-linked anti-rabbit secondary antibody (GE Life Sciences, Buckinghamshire, UK) and then rinsed and exposed with West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) before visualization with PhosphorImager (Storm 860; GE Healthcare, Buckinghamshire, UK).

Propidium iodide staining

Leaf discs (2.5 cm²) from stage C cacao leaves were vacuum-infiltrated using three 2-min applications of 100 µL each (sufficient to cover a leaf disc) of 30 µg/mL propidium iodide (PI), 0.02% Silwet in dH₂O. Mock-treated samples were prepared the same way, but were infiltrated with 100 µL dH₂O containing 0.02% Silwet without PI. To serve as a positive control for dead tissue, a set of leaf discs was infiltrated with 1 mM CuSO₄ 30 min prior to staining with propidium iodide. Leaf discs were mounted in water adaxial side up and imaged on a Zeiss AxioObserver (Carl Zeiss Microscopy GmbH, Jena, Germany) spinning disc confocal microscope, with a 561-nm excitation beam, 617/73 emission filter set and 20× objective. Z-stacks were generated that included the entire depth of the first epidermal cell layer, with 0.2 µm distance between slices. The same laser power, exposure time and detector gain were used for every slice and every sample.

Verification of transgene expression

To measure the levels of transcripts spanning the PI-P-binding domains and EGFP regions of the constructs, RNA was extracted from stage C leaves from stable transgenic lines or, in the case of transient expression experiments, from stage C Scavina6 leaves 5 days following infiltration with *A. tumefaciens* cells. In the case of transient expression experiments, the right-hand side of each leaf was inoculated with *P. tropicalis*, while the left side of each leaf, which was taken for RNA analysis, was mock-inoculated; this ensured that transcript levels were measured under the same conditions as the pathogen assays. RNA was extracted from the leaves using Plant RNA Reagent (Invitrogen) according to the manufacturer's protocol. cDNA synthesis was performed using the New England Biolabs (Ipswich, MA) cDNA Synthesis Kit. Transcript levels were measured by quantitative real-time PCR on a Step One Plus Real-Time PCR System (Applied Biosystems, Waltham, MA) with Takara SYBR Green reagent. PCR cycles were performed at 94 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min. Transcript levels from each transgene were measured relative to the transcript levels of *TcACP1* (Tc01g039970) and *TcTubulin1* (Tc06g000360) of each sample. Primer sequences are listed in Table S1.

To verify transgene insertion in the stable transformants, genomic DNA was extracted from cacao leaves using a modified CTAB method. Stage C leaves were frozen in liquid nitrogen and ground with a mortar and pestle with extraction buffer [10 mM Tris-HCl pH 8.0; 1.4 M NaCl, 10 mM Na₃EDTA pH 8.0, 2% polyvinylpyrrolidone (PVP), 2% cetyltrimethylammonium bromide (CTAB) and 0.2% β-mercaptoethanol] and then further homogenized using a TissueLyzer. Nucleic acids were separated using chloroform:isoamyl alcohol (24 : 1) and precipitated using isopropanol. Pellets were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and treated with RNase A (10 µg/mL) for 30 min at 37 °C, then re-extracted with phenol: chloroform: isoamyl alcohol (25 : 24 : 1) and precipitated using 2.5 M ammonium acetate and 70% ethanol. Resulting pellets were dissolved in sterile dH₂O, and the quantity and quality were checked using a Nanodrop spectrophotometer (Thermo Scientific). A total of 10 ng of each sample was amplified using all three sets of primers (*T. cacao* actin, Bin19 and EGFP, listed in Table S1) at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. Prior to termination, the samples were incubated at 72 °C for 7 min. Samples (20 µL) of each reaction were analysed by electrophoresis on a 2% agarose gel.

Pathogenicity assays

Oomycete pathogens *P. tropicalis* and *P. palmivora* were grown on 20% unclarified V8 medium (100 mL/L V8 juice, 3 g/L calcium carbonate and 15 g/L bacto agar) for 2 days at 27 °C, 12-h daylight. Stage C Scavina6 cacao leaves were inoculated, abaxial side up, on the right-hand side with three agar plugs containing actively growing *Phytophthora* mycelium from the margin of the colony, while the left-hand side was inoculated with sterile agar plugs as a negative control. Inoculated leaves were incubated at 27 °C and 12-h daylight cycle for 3 days before the evaluation of disease symptoms. The leaves were photographed with a Nikon D90 camera (Nikon, Tokyo, Japan) and lesion sizes were measured using ImageJ software tools (Imagej.nih.gov). Average lesion sizes were calculated from three replicates of 18 measurements each, and significance was determined by single-factor ANOVA.

Colletotrichum theobromicola isolates (11–50 and 11–183) were grown on potato dextrose agar (Sigma-Aldrich, St. Louis, MO) for 8 days at 27 °C and 12-h daylight. For inoculations, spores were suspended in sterile deionized water with 0.02% Tween-20, and the concentration was adjusted to 10⁶ spores/mL with a hemocytometer. Leaf pieces, abaxial side up, were inoculated with three 10-µL drops on the right side, while three drops of deionized water were placed on the left side as control. Inoculated leaves were incubated at 27 °C and 12-h daylight for 4 days. Leaves were photographed and lesion sizes were measured as described above.

To assay pathogen DNA as a measure of virulence, the ratio of *Phytophthora* or *Colletotrichum* DNA to cacao DNA was determined by qPCR as follows. Tissue samples including the lesions (1.4 cm² for *P. tropicalis* and *C. theobromicola*, and 2.5 cm² for *P. palmivora* surrounding the inoculation site) were excised from the infected leaves and used for genomic DNA extraction. Tissue was ground using a TissueLyzer homogenizer followed by DNA purification with DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). DNA qPCR was performed as described (Wang *et al.*, 2011) using an ABI 7300 Real-Time PCR System (Applied Biosystems). The relative amount of *Phytophthora* or *Colletotri-*

chum genomic DNA in leaf discs was measured by amplification of the single-copy *PcActin* (BT031870.1), *CtTubulin* (KC512191.1) and *TcActin* (Tc01t010900) genes (Table S1), and the ratio of *Phytophthora* or *Colletotrichum* to cacao DNA was calculated as two to the power of the difference between Ct numbers.

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Conflict of interest

The technology described in this manuscript is the subject of invention disclosures and patent applications by the authors. No other specific conflicts exist.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Transient expression of apoplast-targeted PI3P-binding domain proteins unfused to EGFP also confers resistance to *P. tropicalis* in cacao.

Figure S2 Western blot of total proteins extracted from fresh tissue of nontransformed and stable transformed stage C cacao leaves.

Figure S3 Characterization of stable transgenic cacao plants expressing PI-binding domain proteins.

Table S1 The list of primers used in this study.