Interspecies gene transfer provides soybean resistance to a fungal pathogen

Caspar Langenbach1, Holger Schultheiss2, Martin Rosendahl1, Nadine Tresch2, Uwe Conrath1,* and Katharina Goellner1,*

1 Department of Plant Physiology, RWTH Aachen University, Aachen, Germany
2 BASF Plant Science Company GmbH, Agricultural Center, Limburgerhof, Germany

Summary

Fungal pathogens pose a major challenge to global crop production. Crop varieties that resist disease present the best defence and offer an alternative to chemical fungicides. Exploiting durable nonhost resistance (NHR) for crop protection often requires identification and transfer of NHR-linked genes to the target crop. Here, we identify genes associated with NHR of Arabidopsis thaliana to Phakopsora pachyrhizi, the causative agent of the devastating fungal disease called Asian soybean rust. We transfer selected Arabidopsis NHR-linked genes to the soybean host and discover enhanced resistance to rust disease in some transgenic soybean lines in the greenhouse. Interspecies NHR gene transfer thus presents a promising strategy for genetically engineered control of crop diseases.

Introduction

Host invasion is essential to most pathogens (Panstruga, 2003). During evolution some parasites overcome basal host immunity and adapted to their host, whereas nonadapted pathogens still fail to enter potential host cells (Heath, 2000). Nonhost resistance (NHR) of plants is remarkably durable and effective, probably because it utilizes pre- and postinvasion defence responses (Heath, 2000; Lipka et al., 2008). One fundamental question in plant biology is whether NHR to a given pathogen in one species can provide immunity to that pathogen in an otherwise susceptible species.

Knowledge of plant NHR has significantly advanced upon discovery of the PENETRATION (PEN) genes providing preinvasion resistance to nonadapted fungal pathogens in Arabidopsis thaliana (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). Unlike wild-type Arabidopsis, mutants pen1, pen2 and pen3 allow invasion of nonadapted fungal pathogens. They include the powdery mildew fungi Blumeria graminis f. sp. hordei (Bgh) (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006) and Erysiphe pisi (Lipka et al., 2005) and the Asian soybean rust fungus Phakopsora pachyrhizi (Loehrer et al., 2008). phytoalexin-deficient (PAD) 4 and senescence-associated gene (SAG) 101 contribute to postinvasion resistance of Arabidopsis to these fungi. Consequently, the pen2 pad4 sag101 triple mutant, but not single mutant pen2 with intact postinvasion NHR (Langenbach et al., 2013; Lipka et al., 2005), facilitates sporulation of Bgh and E. pisi. pen2 pad4 sag101 also allows enhanced formation of P. pachyrhizi haustoria, which are characteristic feeding organs of plant parasitic fungi (Langenbach et al., 2013). NHR also involves activity of genes in the phenylpropanoid pathway of plant secondary metabolism and key hormones of plant immunity such as salicylic acid and jasmonate (Langenbach et al., 2013; Loehrer et al., 2008; Mellersh and Heath, 2003; Shafiei et al., 2007).

All commercial soybean (Glycine max) varieties are susceptible to Asian soybean rust, the most destructive fungal disease of soybean, commonly causing yield losses of over 50% (Goellner et al., 2010; Hartman et al., 2005). If applied swiftly, fungicides help, but crop losses are still significant. Resistance traits for breeding or genetic engineering of commercial varieties present the best defence. Because of the durability and efficiency of NHR, interspecies transfer of NHR-linked genes from the Arabidopsis nonhost to commercial soybean varieties could present a promising approach for genetically engineered control of Asian soybean rust disease. A similar strategy provided NHR to bacterial streak disease in rice by transfer of the maize Rxo1 resistance gene (Zhao et al., 2005).

Using comparative, genomewide transcriptome profiling we recently identified the gene for UDP glycosyltransferase 84A2 (UGT84A2 or bright trichomes (BRT) 1) as a critical component of Arabidopsis postinvasion NHR to P. pachyrhizi (Langenbach et al., 2013). Here, we extended our analysis to genes transcriptionally coregulated with BRT1. By doing so, we identified ten novel postinvasion NHR-linked Arabidopsis genes. We analysed their contribution to Arabidopsis NHR by gene silencing and demonstrated that some of them provide Asian rust resistance to soybean plants in the greenhouse. Thus, we verify that interspecies transfer of novel NHR-associated genes has the potential for conferring crop resistance to a major fungal disease in the field.

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Results

Identifying NHR-associated genes by comparative transcriptome analysis

We used Affymetrix GeneChips for performing a genomewide analysis of the transcriptional response to *P. pachyrhizi* infection in *Arabidopsis* genotypes impaired to different extents in NHR to Asian soybean rust disease (Langenbach et al., 2013). We compared the transcriptional profiles of wild-type (Col-0), *pen2*, and *pen2 pad4 sag101* plants at the second day postinoculation (d.p.i.) with *P. pachyrhizi* or upon mock treatment (because we analysed only two biological replicates, we did not display microarray data). While the wild type has intact NHR to *P. pachyrhizi*, the *pen2* mutant is impaired in preinvasion NHR to the fungus. *pen2* allows growth of fungal hyphae in the mesophyll but displays postinvasion NHR to soybean rust disease (Langenbach et al., 2013). In contrast to wild type and *pen2*, the *pen2 pad4 sag101* triple mutant has impaired pre- and postinvasion NHR to *P. pachyrhizi* (Langenbach et al., 2013). We reasoned that genes with activated expression in rust-infected *pen2*, but not in infected *pen2 pad4 sag101* or wild type, putatively contribute to postinvasion NHR of *Arabidopsis* to soybean rust disease.

In our analysis, we disregarded genes with altered expression in mock-inoculated plants of any genotype to exclude genes with altered expression due to genetic background and/or mock treatment. By doing so, we identified the *BRT1* gene being essential to *Arabidopsis* postinvasion NHR to *P. pachyrhizi* (Langenbach et al., 2013). Here, we expanded our analysis to genes coexpressed with *BRT1* exclusively in the *P. pachyrhizi*-infected *pen2* mutant. By doing so, we identified ten novel genes putatively contributing to *Arabidopsis* postinvasion NHR to rust disease. We verified their exclusive expression in infected *pen2* by qRT-PCR analysis (Figure 1 and Table 1) and referred to them as postinvasion-induced nonhost resistance genes (PINGs). PING1/7/8 and 10 encode putative hydrolases (Table 1). No other section of the GO category ‘molecular function’ was overrepresented among the ten candidate genes for postinvasion NHR. Intriguingly, half of the PING-encoded proteins contained an N terminal signal peptide (TargetP; http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000). They likely localize to the apoplast (PING2/7/9/10) or contain an apoplast-localized region (PING5) (GO ‘cellular component’). Analysis with the subcellular localization tool for plant proteins ‘softberry Protcomp 9.0’ (http://linux1.softberry.com), correctly predicting 86% of extracellular proteins (Klee and Ellis, 2005), even assigned eight PINGs (PING1/3/4/6/7/8/9/10) to the apoplast and PING2 and PING5 to the plasma membrane. The predicted localization of novel PING-encoded proteins corresponds with their assumed contribution to postinvasion NHR in the mesophyll apoplast.

PINGs essential to *Arabidopsis* postinvasion NHR to *Phakopsora pachyrhizi*

To assess their possible contribution to postinvasion NHR against *P. pachyrhizi*, we used double-stranded RNA interference (dsRNAi) to silence expression of individual PING genes in the *pen2* genetic background (Figure 2 and Table S1). We analysed transformants with strong target gene silencing (>70% reduction in mRNA transcript abundance) by quantitative microscopy in terms of haustoria formation in mesophyll cells. Efficient silencing was achieved for all PINGs (Figure 2a and Table S1), except for PING2 encoding cinnamic acid 4-hydroxylase (C4H) with a role in phenylpropanoid metabolism. We obtained no viable *pen2 PING2-RNAi* transformant. This finding agrees with an earlier report on significant knock-down or loss-of-function of the C4H gene severely affecting plant development or even causing lethality (Schilmiller et al., 2009). RNAi lines with attenuated postinvasion NHR (as determined by enhanced haustoria formation in mesophyll cells) in the first screening (analysis of >300

![Figure 1](image-url) Verification of *Phakopsora pachyrhizi*-induced PING1-10 activation in *pen2*. Relative accumulation of mRNA transcript in wild-type *Arabidopsis* (wt), *pen2*, and *pen2 pad4 sag101* at 2 d.p.i. with *P. pachyrhizi* (+) or after mock treatment (−). We determined PING1-10 transcript abundance in *Arabidopsis* genotypes by qRT-PCR and normalized to *ACTIN2* mRNA. We show the average relative expression of three independent experiments ±SD.
interaction sites on three different leaves) were analysed in more detail (for an overview of all screened RNAi lines, see Table S1). They comprised pen2 PING4, pen2 PING5, pen2 PING6 and pen2 PING9. PING4 encodes a phospholipase-like protein, and PING5 codes for a putative leucine-rich repeat protein kinase. PING6 carries information for an ankyrin repeat family protein, whereas PING9 encodes a putative germin-like protein (Table 1). Each of the four pen2 PING4567 dsRNAi lines was inspected at 800–4500 interaction sites of at least three independent transformation events with >70% gene silencing in generation T1 and/or T2. Silencing in pen2 background of PING4 and PING5 led to approximately threefold, and silencing of PING6 and PING9 to approximately twofold enhanced haustoria percentage when compared with the inoculated pen2 mutant (Figure 2a,b and Table S1).

We confirmed PING5 importance to postinvasion NHR by analysing a homozygous pen2 ping5 double mutant that we created via crossing pen2 with the PING5 promoter T-DNA insertion line SAIL910A07. Similar to the pen2 PING5 RNAi line, pen2 ping5 double mutant displayed approximately 80% reduction in PING5 mRNA transcript abundance when compared with pen2 following inoculation of both genotypes with P. pachyrhizi. Consistent with the increased haustoria presence in the pen2 PING5 RNAi line (Figure 2b and Table S1), pen2 ping5 double mutant had similarly reduced postinvasion NHR (Figure 2c). Together, our findings point to PING4567 and 9 as genes important to postinvasion NHR of Arabidopsis to P. pachyrhizi.

Interspecies transfer of PINGs confers Phakopsora pachyrhizi resistance to soybean

To see whether transfer of Arabidopsis PINGs to soybean would or would not confer resistance to P. pachyrhizi, we stably overexpressed individual PINGs in susceptible soybean cultivar Williams 82 (W82). At 14 days after P. pachyrhizi infection of plants in the greenhouse, we measured the diseased leaf area of 6–12 individual transformation events of PING overexpressors in the T1 generation (three plants per transformation event). Only progeny of transgenic lines with >50% reduction in diseased leaf area in the five best performing transformation events of the first screening (Figure 3) was carried on to the T2 generation and assayed again for resistance or susceptibility to Asian soybean rust disease (Figures 4 and S1). We assessed soybean rust resistance of three transgenic events of W82 PcUBI::PING2/3/4/5/7 and 9. We did not detect P. pachyrhizi resistance in the W82 PcUBI::PING2/3/4/5/7 and 9, even upon careful inspection, did not markedly affect plant phenotype but reproducibly conferred P. pachyrhizi resistance to W82 in the greenhouse. Average reduction in diseased leaf area of PING-expressing plants was 30%–60% (Figure 4). The increase in P. pachyrhizi resistance was significant for all analysed events of W82 PcUBI::PING4 and W82 PcUBI::PING7 and for two of three analysed events of W82 PcUBI::PING5 (Figure 4). In contrast, none of the examined events of W82 PcUBI::PING2 and W82 PcUBI::PING3 was more resistant to rust disease than the nontransgenic W82 control (Figures 4 and S1). Except W82 PcUBI::PING9A, the analysed transgenic events strongly expressed the corresponding PING transgene (Figure 4). We did not detect expression of PING9 in W82 PcUBI::PING9A suggesting plants being azygous (Figure 4). Consistent with transgene expression, only W82 PcUBI::PING9B and W82 PcUBI::PING9C, yet not W82 PcUBI::PING9A, had enhanced resistance to P. pachyrhizi (Figure 4). In sum, overexpression of PING457 and 9 reproducibly diminished rust symptoms on soybean W82.

<table>
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<th>Proposed name</th>
<th>Identifier</th>
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<th>Encoded protein</th>
<th>Fold change wild type (P.p./mock)</th>
<th>Fold change pen2 (P.p./mock)</th>
<th>Fold change pen2 pad4 sag101 (P.p./mock)</th>
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AGI, Arabidopsis Genome Initiative; P.p., Phakopsora pachyrhizi.

Listed genes were activated at 2 d.p.i. with P. pachyrhizi in pen2, but not, or only scarcely, in pen2 pad4 sag101 or wild type. Table entries are sorted from highest to lowest induction in pen2 as detected in microarray analysis. Fold change in gene expression in P. pachyrhizi-inoculated versus mock-treated genotypes is shown. Expression values of BRT1 (Langenbach et al., 2013) are given for reference.
We tested the enhanced resistance of W82 PcUBI::PING4/5/7/9 to soybean rust disease with a second method (Figure 5a). In addition to providing greater sensitivity, the assay also provides information on the infection stage at which transgene expression antagonizes fungal development. We used qRT-PCR for determining, over time, the ratio of fungal mRNA transcript to plant mRNA transcript in leaves of the P. pachyrhizi-infected wild type (W82) and in two events of PING4/5/7/9 overexpression lines (Figure 5a). An increase in ratio indicates continuing fungal growth (van de Mortel et al., 2007). We discovered increasing levels of fungal mRNA transcript from 1 to 7 d.p.i., but only small changes from 7 to 14 d.p.i., in the wild type (Figure 5a). We detected a similar increase in fungal mRNA in the transgenic lines (Figure 5a), which all expressed PING transgenes at high levels (Figure 5b). However, in the transgenic lines, the increase in fungal mRNA in most cases was lower than in wild type (Figure 5a) with significant differences seen as early as 2–3 d.p.i. when haustoria developed in the mesophyll (Figure 5a,e,f,g). Thus, PING4/5/7 and 9 obviously support postinvasion resistance against P. pachyrhizi, at least in the W82 soybean host. In line with the reduced symptom development in the transgenic lines (Figure 4), PING4/5/7 and PINGS overexpression lines showed 85%–95% and approximately 65% reduced accumulation of fungal mRNA transcript at 14 d.p.i., respectively (Figure 5a). Hence, expression of Arabidopsis PING4/5/7 or 9 in W82 soybean attenuates fungal colonization of the leaf mesophyll and reduces rust disease symptoms on the soybean host.

Figure 2 PING4/5/7/9 silencing in pen2 attenuates Arabidopsis postinvasion nonhost resistance (NHR) to Phakopsora pachyrhizi. (a) dsRNAi-mediated silencing of PING4/5/7/9 expression in transgenic pen2 as compared to untransformed pen2. (b) Haustoria frequency in P. pachyrhizi-infected pen2 without or with PING4/5/7/9 silencing at 2 d.p.i. We determined average haustoria frequency (+SD) by quantitative microscopy of approximately 4000 penetration events in pen2 (control) and of 800–4500 penetration events in at least three independent dsRNAi lines of the T1, and/or T2 generation. Table S1 provides detailed information on postinvasion NHR of all tested pen2 PING-RNAi lines and gives numbers of analysed interaction sites. (c) We verified function of PING5 in postinvasion NHR by determining haustoria frequency in pen2 ping5 double mutants. Average haustoria frequency (+SD) of three independent experiments is shown. We evaluated approximately 1300 penetration events for each genotype.

Figure 3 Reduced appearance of disease symptoms in soybean (W82) lines overexpressing Arabidopsis PINGS. Shown is the relative average reduction in diseased leaf area of the five best performing transgenic lines in the T1 generation compared to W82 control (average value) at 14 d.p.i. with Phakopsora pachyrhizi. Only lines with >50% symptom reduction (dotted line indicates cut-off) were propagated to assess soybean rust disease resistance in the T2 generation (Figures 4 and S1).

We tested the enhanced resistance of W82 PcUBI::PING4/5/7 and 9 to soybean rust disease with a second method (Figure 5a). In addition to providing greater sensitivity, the assay also provides information on the infection stage at which transgene expression antagonizes fungal development. We used qRT-PCR for determining, over time, the ratio of fungal mRNA transcript to plant mRNA transcript in leaves of the P. pachyrhizi-infected wild type (W82) and in two events of PING4/5/7/9 overexpression lines (Figure 5a). An increase in ratio indicates continuing fungal growth (van de Mortel et al., 2007). We discovered increasing levels of fungal mRNA transcript from 1 to 7 d.p.i., but only small changes from 7 to 14 d.p.i., in the wild type (Figure 5a). We detected a similar increase in fungal mRNA in the transgenic lines (Figure 5a), which all expressed PING transgenes at high levels (Figure 5b). However, in the transgenic lines, the increase in fungal mRNA in most cases was lower than in wild type (Figure 5a) with significant differences seen as early as 2–3 d.p.i. when haustoria developed in the mesophyll (Figure 5a,e,f,g). Thus, PING4/5/7 and 9 obviously support postinvasion resistance against P. pachyrhizi, at least in the W82 soybean host. In line with the reduced symptom development in the transgenic lines (Figure 4), PING4/7/9 and PINGS overexpression lines showed 85%–95% and approximately 65% reduced accumulation of fungal mRNA transcript at 14 d.p.i., respectively (Figure 5a). Hence, expression of Arabidopsis PING4/5/7 or 9 in W82 soybean attenuates fungal colonization of the leaf mesophyll and reduces rust disease symptoms on the soybean host.
Discussion

We report the transfer of certain Arabidopsis immunity genes provides resistance to Asian soybean rust disease in soybean plants in the greenhouse. Earlier reports demonstrated the feasibility of gene transfer approaches for crop protection. For example, Zhao et al. (2005) conferred enhanced resistance to the bacterium Xanthomonas oryzae pv. oryzae to rice by within-family (Poaceae) transfer of the maize Rbo1 resistance (R) gene. Similarly, the within-species transfer of three combined quantitative trait loci from resistant wild lettuce provided enhanced immunity against the oomycete Bremia lactucae to cultivated lettuce (Zhang et al., 2009). In contrast to these and similar earlier reports, our approach rests upon the interspecies transfer of NHR-linked genes for providing soybean resistance to a fungal pathogen, which is one of the most serious threats to food security (Pennisi, 2010). In addition, earlier reports exploited the transfer of either microbial pattern recognition receptors (PRRs) (Lacombe et al., 2010) or classical R genes (Tai et al., 1999; Zhao et al., 2005) for providing disease resistance to crops that normally fail recognizing a given pathogen. However, R gene-mediated resistance might be less durable than NHR, which is quantitative and seems to rely on complex action of multiple genes and loci (Ellis, 2006; Fan and Doerner, 2012). Thus, exploitation of NHR likely reduces the risk of the pathogen overcoming resistance. Moreover, the parasite will likely need longer to conquer resistance originating from an unrelated plant than from the same species. The stacking of multiple genes contributing to NHR is likely to further increase immunity and reduce the risk of resistance breaks.

Of the ten identified PINGs, only PING4/5/9 seem to be essential to Arabidopsis postinvasion NHR to P. pachyrhizi (Figure 2) and to provide rust resistance upon transfer to the soybean host (Figure 4). In contrast, transfer of the PING6 gene required for postinvasion NHR to P. pachyrhizi in Arabidopsis (Figure 2a,b) did not enhance rust resistance in soybean (Figure 3). The latter finding indicates inefficiency of the PING6-encoded Arabidopsis ankyrin repeat family protein in soybean.

The opposite is true for PING7 whose expression as a transgene in soybean quantitatively enhanced resistance to soybean rust (Figure 4). Yet, PING7 silencing in Arabidopsis did not enhance susceptibility to P. pachyrhizi (Table S1), possibly because of functional redundancy within the big GDSL-motif lipase/hydrolase gene family (Ling, 2008).

According to their GO annotation, three (PING S/7/9) of the four (PING4/5/7/9) genes with capacity to provide immunity to P. pachyrhizi in soybean are predicted to encode proteins that either localize to the apoplast or do contain an extracellular, putatively carbohydrate binding malectin-like domain (Schallus et al., 2008). Therefore, we speculate that PING 5, 7 and 9 contribute to prehaustorial postinvasion resistance in the apoplast by either impairing hyphal integrity or antagonizing haustoria formation. Both these effects could be mediated by antifungal (e.g. by GDSL lipases; Oh et al., 2005) or cell wall-reinforcing proteins (e.g. by germin-like proteins; Christensen et al., 2004; Schweizer et al., 1999; Zimmermann et al., 2006), leucine-rich repeat receptor-like kinases (LRR-RLKs) or combinations of these proteins. The LRR-RLK might recognize the invading pathogen, or signals released by the pathogen in the apoplast and mediate signalling into the cell to ultimately activate defence and provoke immunity.

Although their exact mode of action remains elusive, genes for early Arabidopsis aluminium-Induced (EARLI) 4-like phospholipase (Richards et al. 1998) (PING4) [note EARLI4 has been annotated as PEARLI4 at The Arabidopsis Information Resource], group I receptor-like kinase (PING5), GDSL-like lipase (PING7) and the germin-like protein (PING9) all confer P. pachyrhizi resistance to soybean (Figures 4 and 5). They belong to gene families so far associated with plant defence to pathogens other than P. pachyrhizi (Chen et al., 2014; Christensen et al., 2004; Hok et al., 2011; Humphry et al., 2010; Kwon et al., 2009; Oh et al., 2005). The Arabidopsis earl4 mutant is hypersusceptible to the powdery mildew fungus Golovinomyces orontii and attenuated in its NHR to E. pisi (Humphry et al., 2010). GDSL-like lipase 1 (GLIP1) is important for Arabidopsis immunity to the fungus Alternaria brassicicola and various bacterial pathogens (Oh et al., 2005). GLIP1 is an extracellular protein crucial to local and systemic immune signalling, but also having direct antifungal activity (Kwon et al., 2009). Germin-like proteins are important to resistance against various plant pathogenic fungi and insect pests (Dunwell et al., 2008). Impaired Oomycete Susceptibility (IOS1), a member of group I LRR-RLKS, is associated with the PRRs flagellin-sensing 2 (FLS2) and elongation factor-Tu receptor (EFR) and required for priming of pattern-triggered immunity (Chen et al., 2014). In the interaction of Arabidopsis with the oomycete pathogen Hyaloperonospora arabidopsidis, IOS1 serves as a susceptibility factor (Hok et al., 2011). Thus, the here identified NHR-linked genes represent so far unnoticed members of known defence gene families.

PING4/5/9 are essential for Arabidopsis postinvasion NHR to P. pachyrhizi and expression of PING4/5/7/9 in the soybean host affects fungal proliferation as early as 2–3 d.p.i. (Figure 5a). At this time, the fungus has successfully invaded the host mesophyll and haustoria start to develop (Figure 5e–g). Thus, identified genes indeed likely encode proteins of the postinvasion defence machinery that are functional in the nonhost and the host. It will be interesting to learn whether transfer of the previously identified postinvasion NHR gene BRT1 (Lagenbach et al., 2013) will also provoke P. pachyrhizi resistance in soybean. For unknown reasons, we did not succeed in creating soybean plants expressing Arabidopsis BRT1 as a transgene so far.

Together, we here pursued a strategy that provided engineered resistance to Asian soybean rust disease in the soybean crop in the greenhouse. On the long term, the strategy may complement current breeding programmes and fungicide applications for providing soybean rust resistance in the field. Field-site experiments with soybean plants expressing individual or multiple transgenes under control of optimized promoters and at natural disease pressure will clarify the agronomic value of engineered soybean varieties.

Experimental procedures

Arabidopsis genotypes and fungal material

For creating the pen2-1 pad4-1 sag101 triple mutant, sag101 T-DNA insertion line (N661816; SALK_022911) was crossed to pen2-1 pad4-1 double mutant. We produced the pen2 ping5 double mutant by crossing pen2-1 to the ping5 T-DNA insertion line (N840993; SAIL910A07). Wild-type Arabidopsis and mutants were grown and P. pachyrhizi (isolate Br05) propagated as described (Langenbach et al., 2013). Inoculation of Arabidopsis
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with Phakopsora pachyrhizi was also performed as reported previously (Langenbach et al., 2013).

Creation of transgenic Arabidopsis plants

To provide dsRNAi constructs for stable silencing of PING2/3/4/5/7 and 10 in Arabidopsis, full-length or partial sequences were Gateway® cloned in the plawohl vector (GenBank Accession Number AF408413). We obtained PING3/4 and 7 cDNA from the Arabidopsis Biological Resource Center (ABRC; CATMA1A23520; CATMA2a15560, CATMA5A02790) whereas PING2/5 and 10 were PCR-amplified from wild-type Arabidopsis (accession Col-0) genomic DNA using gene-specific primers (Table S2). Sequenced destination vectors were transformed to Agrobacterium tumefaciens strain GV3101 and used for stable Arabidopsis transformation using the floral dip technique (Clough and Bent, 1998). For silencing PING1/6/8 and 9, we sequenced artificial microRNA (amiRNA) clones CSHL_070753; CSHL_058261; CSHL_042284; and CSHL_074851 from ABRC and cotrans-
formed them into A. tumefaciens GV3101, together with pSoup helper plasmid (ABRC number CD3-1124). Transgenic plants were selected on BASTA® (Bayer, 200 g/L glufosinate-ammonium)-drenched soil and analysed by PCR to check T-DNA presence.

Cloning of PING overexpression constructs for soybean transformation

We cloned PING coding sequences first into a Gateway pENTRY vector (Thermo Fisher Scientific, St. Leon-Rot, Germany) and then into a binary pDEST vector (Thermo Fisher Scientific, St. Leon-Rot, Germany) so as to full-length fragments were located, in sense direction, between the parsley ubiquitin promoter (PcUbi) and a potato CAT-ga terminator. pDEST vectors contained a spectinomycin/streptomycin resistance cassette for bacterial selection, a pVS1 origin for replication in Agrobacterium, a pBR322 origin of replication for stable maintenance in Escherichia coli and the selectable marker acetohydroxyacid synthase (AHAS) gene under control of a PcUbi promoter.

Soybean transformation

Transformation of soybean cultivar W82 was carried out as described (Olhoft et al., 2007) with some modifications. Briefly, disarmed Agrobacterium rhizogenes SHA17 harbouring the appropriate pDEST binary vector was grown in YEP growth medium containing 100 mg/L spectinomycin. To prepare the inoculum, agrobacteria were grown at 28 °C in 400 mL YEP medium containing 100 mg/L spectinomycin to an OD660 of 1.0–1.5. Agrobacteria were collected by centrifugation (5000 g, 9 min) and resuspended in co-cultivation medium [0.1 x Gamborg’s BS salts, 30 g/L sucrose, 20 mM MES hydrated, 1 x Gamborg’s vitamins, 5 μM kinetin, 0.5 mg/L gibberellic acid, 0.2 mM acetosyringone (pH 5.4)] to an OD660 of 1.5. The suspension of resuspended agrobacteria was kept at room temperature for at least 30 min before use.

Soybean explants were prepared by removing most of the hypocotyl, one cotyledon and all preformed leaves (including the apical meristem) from 7- to 8-day-old soybean (W82) seedlings. After co-cultivation with the resuspended agrobacteria for 30 min, explants were transferred to petri dishes containing co-cultivation medium (see above, but containing 4.4 mM L-cysteine, 0.5 mM sodium thiosulfate, 0.5 mM DTT). After 5 days at 25 °C in the dark, explants were transferred to Oasis® wedges (Oasis® Grover Solutions, Kent, Ohio, U.S.A.). Explants were placed vertically in the wedges with their residual hypocotyl part inserted into the wedges. Wedges were watered with a solution of 1 μMArsenal® (imazapyr). When shoots became elongated from the infected primary leaf node region, they were separated from the seedling and rooted individually in Oasis® wedges. Detached shoots were watered with a solution containing Arsenal® once or twice a week. When the shoots became rooted, they were transferred to soil and grown to maturity in the greenhouse.

Evaluation of Arabidopsis postinvasion NHR

Arabidopsis leaves were harvested 2 d.p.i. with P. pachyrhizi and subjected to analysis of candidate gene expression and trypan blue staining as described (Langenbach et al., 2013). To evaluate postinvasion NHR, we determined frequencies of haustoria in leaves upon trypan blue staining, chloral hydrate destaining and bright-field microscopy (Leica, Bensheim, Germany). We assessed at least 100 interaction sites per leaf.

Assessing soybean resistance to Phakopsora pachyrhizi

Soybean cultivar W82 grew in a greenhouse at 21–27 °C and 75% humidity. We inoculated the plants when the first trifolium leaf had fully expanded. To do so, we collected P. pachyrhiziuredospores from leaves of severely diseased soybean plants and suspended them in 0.01% (v/v) Tween-20 at a density of 2 × 106 spores per mL. We randomly arranged the various soybean genotypes and inoculated them using a spraying box. After incubation in a dark chamber and at 100% relative humidity for 24 h, we transferred the plants to the greenhouse and grew them at the above conditions until assessment. We rated the diseased area on primary leaves and the first trifolium at 14 d.p.i. In each experiment, we analysed at least three plants of three independently derived transgenic lines (three independent transformation events) to assess resistance to P. pachyrhizi. Overexpression of PING6 took place in soybean cultivar Jake. Nontransgenic plants of this cultivar served as control when analysing soybean rust resistance of the PING6 overexpressors. Fungal mRNA abundance was measured as described (van de Mortel et al., 2007; Pandey et al., 2011). RNA was extracted from infected soybean leaves and reverse transcribed to cDNA as described below.

Microscopic analysis of Phakopsora pachyrhizi-infected soybean leaves

Leaves were harvested and stained as described (Schneider et al., 2011). Upon destaining in saturated chloral hydrate, leaves were analysed using bright-field microscopy. DISKUS software (Carl H. Hilgers, Königswinter, Germany) served for image editing.

mRNA transcript quantification

Total RNA was extracted from Arabidopsis leaves as described (Chomczynski and Sacchi, 1987). We extracted soybean RNA by mixing approximately 100 mg ground, frozen leaf material with 600 μL of lysis buffer (2%, w/v, ultrapure SDS, 68 mM trisodium citrate, 132 mM citric acid, 1 mM EDTA, pH ~3.5). Then, protein was settled by adding 200 μL precipitation buffer (4 mM NaCl, 17 mM trisodium citrate, 33 mM citric acid, pH ~3.5) and 5 min incubation on ice. After centrifugation at 12,000 g for 5 min and at room temperature in a minifuge, the supernatant was transferred to fresh test tubes and RNA precipitated for 15 min with 600 μL isopropanol. Upon further centrifugation for 5 min in a minifuge, we washed the pellets with 70% (v/v) ethanol, dried shortly and resuspended them in 30 μL RNAse-free ultrapure water. Independent of the origin (Arabidopsis, soybean or P. pachyrhizi), RNA was reverse transcribed to cDNA using 9-mer random primers and RevertAid® reverse transcriptase (Thermo Fisher Scientific, St. Leon-Rot, Germany), as described by the manufacturer. cDNA was used in qRT-PCR with SYBR® Green (Thermo Fisher Scientific, St. Leon-Rot, Germany) to determine the level of obtained gene-specific cDNA. qRT-PCR was carried out as described (Langenbach et al., 2013; van de Mortel et al., 2007). Primers were designed standard (Udvardi et al., 2008) and blasted for putative off target binding using the Primer Blast tool at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Table S2 lists the primers used for mRNA quantification.

GeneChip analysis

For global transcriptome analysis, leaf material of the wild-type Arabidopsis (accession Col-0), and pen2 and pen2 paddy sag101 mutant was collected 2 d.p.i. with P. pachyrhizi or upon mock
treatment. Leaf material served for RNA extraction or trypan blue staining. To verify normal proliferation of the fungus on each Arabidopsis genotype, and to assess whether the fungus had already established hyphae and/or haustoria in the mesophyll of pen2 and pen2 pad4 sag101, trypan blue-stained leaves of each plant were examined by bright-field microscopy. For each genotype and treatment, we pooled leaves of four different plants and used it for RNA extraction following a protocol slightly modified to the one mentioned above: following chloroform extraction, RNA was not precipitated using isopropanol; we rather purified RNA using RNeasy Mini spin columns (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. All steps of labelling, hybridization with Affymetrix ATH1 GeneChips and scanning were carried out at the IZKF (Münster, Germany). Only samples with RNA integrity number values >8 served for transcriptome analysis. We processed raw expression data of two independent experiments using the Web-based pipeline for microarray gene expression-profile analysis (GEPAS; Herrero, 2003) merged with Babelomics to a unique new web application (http://babelomics.bioinfo.cipf.es/; Medina et al., 2010). Robust multiarray averaging (Irizarry et al., 2003) was chosen for background correction and data normalization. We monitored gene expression using the FiRe program (Garcion et al., 2006). We verified by qRT-PCR abundance of mRNA transcripts in pen2 but no, or only weak, abundance in wild type or pen2 pad4 sag101 in both replicates of three additional experiments. We selected only genes with consistent activation in all replicates for functional analysis.

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Conflict of interest

All authors except M.R. are inventors of linked patent WO/2013/093738. H.S. and N.T. are employees of BASF Plant Science Company GmbH.

References


Supporting information

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

**Figure S1** Transfer of *Arabidopsis PING2* gene to soybean W82 does not enhance resistance to Asian soybean rust disease.

**Table S1** Gene silencing efficiency and haustoria formation frequency in *ping2* *PING*-RNAi lines 2 days after inoculation with *Phakopsora pachyrhizi*.

**Table S2** Primer sequences used for cloning and for qRT-PCR.