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Expression analysis reveals a role for hydrophobic or epicuticular wax signals in pre-penetration structure formation of *Phakopsora pachyrhizi*

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Asian soybean rust (ASR) caused by the fungus *Phakopsora pachyrhizi* is one of the most devastating foliar diseases affecting soybean production worldwide. Even though several resistance sources have been identified in soybean, they do not show resistance to all races of *P. pachyrhizi*. Identification of genes that confer nonhost resistance (NHR) against *P. pachyrhizi* in another legume species will provide an avenue to engineer soybean to have durable and broad spectrum resistance against *P. pachyrhizi* strains. Recently, we identified a *Medicago truncatula* gene, *IRG1* (*INHIBITOR OF RUST GERM-TUBE DIFFERENTIATION1*), that when mutated inhibits the growth of *P. pachyrhizi*. *IRG1* encodes a Cys(2)His(2) zinc finger transcription factor that controls wax-biosynthesis-related genes. The *irg1* mutant shows a complete loss of abaxial epicuticular wax crystals and surface hydrophobicity, resulting in the inhibition of pre-penetration structure formation. In order to confirm the role of surface hydrophobicity in the formation of pre-penetration structures, we examined the expression profiles of *P. pachyrhizi* putative pre-penetration structure-development-related genes on a solid surface or a *M. truncatula* abaxial leaf surface. Interestingly, the expression of kinase family genes was upregulated on the hydrophobic surface and *M. truncatula* wild-type leaf surface, but not on the *M. truncatula irg1* mutant leaf surface, suggesting that these genes play a role in *P. pachyrhizi* pre-penetration structure development. In addition, our results suggest that hydrophobicity on the *M. truncatula* leaf surface may function as a key signal to induce the *P. pachyrhizi* genes involved in pre-penetration structure development.

Asian soybean rust (ASR) caused by biotrophic plant pathogenic fungus, *Phakopsora pachyrhizi* is one of the devastating diseases of soybean. The disease cycle of *P. pachyrhizi* begins with urediniospores, which have an important role in the disease cycle. The urediniospores attach to the surface of host leaves and produce pre-penetration structures, including germ tubes and appressoria. Unlike other rust pathogens, *P. pachyrhizi* is a unique, directly penetrating rust fungus. After penetration, *P. pachyrhizi* develops infection hyphae, colonizes host cells, and forms a specialized feeding structure called haustorium. *P. pachyrhizi* develops tan lesions on the leaf surface of a susceptible soybean plant one week after infection and then makes uredinia, which are structures that produce urediniospores on the abaxial leaf surface.[1,2] Five soybean resistance genes, *Rpp1–5*, confer immunity or resistance to *P. pachyrhizi*.[3-6] However, there is no soybean line that has broad-spectrum disease resistance to all races of *P. pachyrhizi*.[7] Therefore, the demand for development of durable resistance to *P. pachyrhizi* is high. Understanding the mechanism of plant immunity against *P. pachyrhizi* would benefit the development of durable resistant plants. The nonhost resistance (NHR) is the most common and durable form of resistance against potential pathogens in nature. NHR mechanisms can be utilized for improving resistance to pathogen infection in crop plants.[8-10]

Medicago truncatula, a model plant species for legumes, shows NHR response to *P. pachyrhizi*. It has been demonstrated that *P. pachyrhizi* forms germ tubes with appressoria and penetrates into epidermal cells, resulting in necrotic symptoms without sporulation on *M. truncatula*.[11] To identify mutants that show an altered phenotype to *P. pachyrhizi* infection, a forward genetics screen using *Tnt1* insertion mutant lines of *M. truncatula*[12] has been developed and an *inhibitor of rust germ-tube differentiation1* (*irg1*) mutant that inhibited fungal pre-penetration structure differentiation was identified.[11] Interestingly, *irg1* mutants showed pre-penetration resistance against rust pathogens, including *P. pachyrhizi* and *Puccinia emaculata* (switchgrass pathogen), and the hemibiotrophic anthracnose fungus *Colletotrichum trifolii*, but not to necrotrophic fungal pathogens *Phoma medicagenis* and *Sclerotinia sclerotiorum*.[11] Leaves of *irg1* mutant lack abaxial epicuticular wax crystals, indicating that inhibition of rust pre-infection structures in *irg1* mutant is connected with the loss of surface hydrophobicity. Furthermore,

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Table 1. List of gene-specific primer sets for RT-qPCR of *P. pachyrhizi*

Gene	EST	Forward primer	Reverse primer
Elongation factor 1α	Pp1107	ATACGCTCCT GTCCTTGATT GCCA	AACAGTTTGC CTCATGTCAC GCAC
Ubiquitin 5	Pp1724	AGAGGGAATT CCTCCAGACC AACA	ATTTGCATTC CACCACGAAG TCGG
TKL family protein kinase	Pp1003	AGGAGTGGAA TACTTGCACT TGCG	ATGCTGTGGT TGTAAGACGG GTGA
Chitin synthase	Pp1605	TCTAGGATGG CTAGCCCATT TGGT	GTGATTGTGT TCAAATCCGC CCGT
Calcium/calmodulin protein kinase	Pp0839	TCTGCCGTCG AACACATTCA CTCT	TAAACCTCCG GTGCTGTGTA ACCT
Metacaspase	Pp0322	CAACAAGGCC GACCAAGTTT CCAA	TGCTGATATGTCTGCTGCGG GTTA
Histidine kinase	Pp0948	AGCCATCGAT CTCATCCAAC ACGA	TGTAATGCTC AGCGGTTGAG AGGT
NADH dehydrogenase	Pp1722	TCCGAGCAGC TATGAAACAC TGGT	AAAGCTTGGT GCGCTTGTAG TCTC

we demonstrated that *IRG1* encodes the Cys(2)His(2) zinc finger type transcription factor that regulates wax biosynthetic pathways in *M. truncatula*.[11] To investigate the function of epicuticular waxes in stimulating the differentiation of fungal pre-penetration structures such as germ tubes and appressoria, we performed quantitative analyses of fungal development during in vitro germination assay. First, epicuticular waxes were extracted with hexane. Then, urediniospores of *P. pachyrhizi* were placed on hydrophilic glass surfaces coated with or without epicuticular waxes isolated from both surfaces of *M. truncatula* wild-type and *irg1* mutants, and kept in a high humidity chamber. Although the waxes isolated from the adaxial leaf surface of both wild-type and *irg1* mutant induced the formation of pre-penetration structures compared with the mock (hexane-coated slide glass), there was no significant difference in their ability to induce the pre-penetration structures between wild-type and *irg1* mutants. However, we found a significant reduction in the formation of pre-penetration structures on the glass slides coated with waxes isolated from the abaxial leaf surface of *irg1* mutants compared with wild-type, suggesting that epicuticular waxes or hydrophobicity promote the formation of pre-penetration structures such as germ tube and appressoria.[11]

The formation of pre-penetration structures is a crucial step for the pathogenicity of rust pathogens including *P. pachyrhizi*.[1] Therefore, understanding the molecular basis of mechanisms related to the formation of pre-penetration structures is essential for providing novel strategies for disease management. In other economically important fungal pathogens such as rice blast, several signaling pathways including mitogen-activated protein kinase (MAPK) signaling pathways, G-protein-mediated signaling pathways, calcium/calmodulin-mediated signaling pathways, and cAMP-mediated signaling pathways have been identified. Mutant analyses of genes involved in these signaling pathways have revealed that these pathways are required not only for the formation of pre-penetration structures, but also for the pathogenicity of rice blast.[13] Unlike rice blast, limited information is available for the regulators of pre-penetration structure development on rust pathogens. In order to investigate the regulation of pre-penetration structure development in *P. pachyrhizi*, we examined the expression profiles of *P. pachyrhizi* pre-penetration structure-development-related genes on a solid surface or *M. truncatula* abaxial leaf surface. We selected putative pre-penetration structure-development-related genes, including *chitin synthase*, *kinase family gene*, and *metacaspase*, and housekeeping gene, *NADH dehydrogenase* (Pp1722) from *P. pachyrhizi* expressed sequence tag,[7] and performed RT-qPCR analysis using gene-specific primer sets (**Table 1**). It is interesting that the abaxial leaf surface of the *irg1* mutant showed reduced pre-penetration structure development of *P. pachyrhizi*.[11] Therefore, one could argue that the regulation of pre-penetration structure-development-related genes in the *irg1* mutant may result from reduced viability of urediniospores rather than from the direct effects of host signals such as epicuticular waxes or hydrophobicity. To rule out this possibility, we investigated the expression of *NADH dehydrogenase* (Pp1722), and found no significant differences in the gene expression between *M. truncatula* wild-type and the *irg1* mutant. The expression of *kinase family genes*, including *TKL family protein kinase* (Pp1003) and *calcium/calmodulin-dependent protein kinase* (Pp0839), *chitin synthase* (Pp1605), and *metacaspase* (Pp0322) was upregulated on the hydrophobic surface and *M. truncatula* wild-type leaf surface, but not on the *M. truncatula irg1* mutant leaf surface, suggesting that these genes may have a role in pre-penetration structure development in response to epicuticular waxes or hydrophobicity (**Fig. 1**). It has been demonstrated that the calcium/calmodulin-mediated signaling pathway is involved not only in fundamental physiological processes, but also in the pathogenicity of filamentous fungi.[14] It was reported that the expression of *calcium/calmodulin-dependent protein kinase* was upregulated in response to the hard surface contact in rice blast and anthracnose pathogens.[15,16] These results suggest that *P. pachyrhizi* also utilizes the calcium/calmodulin-mediated signaling pathway to regulate pre-penetration structure development. The expression of *histidine kinase* (Pp0948) was downregulated on the hydrophobic surface and *M. truncatula* wild-type leaf surface compared with the *M. truncatula irg1* mutant leaf surface, suggesting that histidine kinase may have negative functions on pre-penetration structure development. Histidine kinases are known as important mediators for adaptation to stresses from prokaryotes to eukaryotes.[17,18] In addition, histidine kinases have been demonstrated to have an impact on the pathogenicity of fungal pathogens such as *Botrytis cinerea*, *Fusarium oxysporum*, *Claviceps purpurea*, and rice blast.[19-22] Interestingly, histidine kinase was reported to impact the pathogenicity of *Alternaria longipes*. It has been demonstrated that the *A. longipes histidine kinase* (*AlHK1*)-null mutant produced larger lesions compared with the wild-type strain, suggesting that histidine kinase functions as a negative regulator for the pathogenicity of *A. longipes*.[23] Taken together, histidine kinase (Pp0948) may be involved in the pathogenicity of *P. pachyrhizi* in a negative manner.

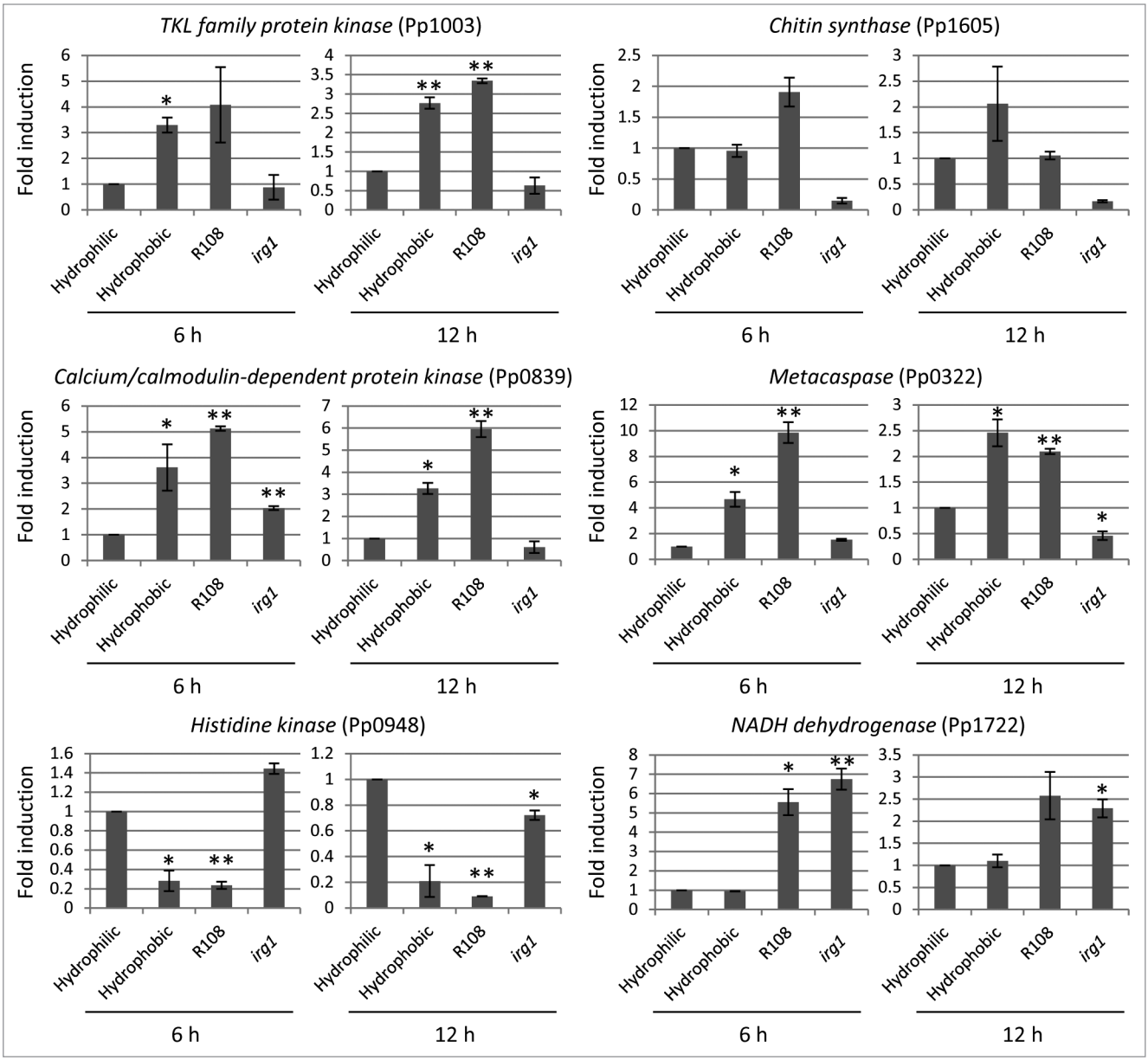

Figure 1. Expression profiles of *TKL family protein kinase* (Pp1003), *chitin synthase* (Pp1605), *calcium/calmodulin-dependent protein kinase* (Pp0839), *metacaspase* (Pp0322), *histidine kinase* (Pp0948), and *NADH dehydrogenase* (Pp1722) during pre-penetration structure development of *P. pachyrhizi*. Fresh urediniospores (1×10^5 spores/ml) were incubated on hydrophilic glass plates and hydrophobic Petri plates, or spot-inoculated on *M. truncatula* wild-type (R108) and *irg1* mutants. The inoculated plates and leaves were incubated in the dark, and then total RNA was isolated using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Five μg of DNase-treated RNA was reverse transcribed, and the cDNA (1:10) was then used for RT-qPCR. The average threshold cycle (C_T) values calculated from triplicate biological samples were used to determine the fold expression relative to the controls. Primers specific for *elongation factor 1α* (Pp1107) and *ubiquitin 5* (Pp1724) were used to normalize differences in template amounts. Vertical bars indicate the standard errors for 3 independent experiments. Asterisks indicate a significant difference from hydrophilic glass plate using a t-test (* = $p < 0.05$, ** = $p < 0.01$).

These results indicate that epicuticular waxes or hydrophobicity may function as key signals for the pre-penetration structure development of *P. pachyrhizi*.

In conclusion, we demonstrated the importance of host signals such as epicuticular waxes and/or hydrophobicity in the pre-penetration structure development of *P. pachyrhizi*. Although the precise role of IRG1 in wax biosynthesis and the mechanism by which hydrophobicity induces fungal genes involved in pathogenicity need further investigation, our results present the possibility of a novel strategy for disease management by manipulating plant surface hydrophobicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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