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Functional analysis reveals an expansive evolution of the *PRLIP* (Pathogenesis Related Lipase) gene family in plants

PhD Thesis

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INTRODUCTION

PR proteins are important effector molecules of plant disease responses. They are commonly found in the plant kingdom and currently classified into 17 PR families based on biological and biochemical properties.

However, no PR family consisting of lipases or lipase-like proteins has been established yet, although recent studies highlight the importance of lipases and lipidic signals in different stress responses of plants. Lipases, along with esterases, belong to the alpha/beta-hydrolase fold family of enzymes. They preferentially hydrolyze long-chain acylglycerols into glycerols and free fatty acids, while the latter primarily hydrolyze short chain acylglycerols. The stress hormone jasmonic acid (JA), a lipid derivative itself, has been proposed to play a role in lipidic signaling in JA wound responses. Salicylic acid (SA) mediated Systemic Acquired Resistance (SAR) is also strongly affected by lipidic signaling through SABP2, a protein having SA-repressed esterase activity, thus acting as a receptor for SA in tobacco. In Arabidopsis, a similar function has been attributed to the homologous AtMES proteins with esterase activity.

In addition to these well characterized proteins plenty of other lipase enzymes have been related to plant stress responses. One group of these stress-related lipases of special interest is the GDSL (also known as SGNH) lipases. GLIP1, a GDSL-type lipase, was identified to be highly induced in response to ethephon treatment and to play an important role in inducible resistance against *Alternaria brassicicola* in Arabidopsis. It might be either implicated in the signaling process or exert antimicrobial activity. Transgenic plants overexpressing GLIP1 exhibited increased defense against *Alternaria* and were assumed to form a GLIP1–ethylene (ET) signaling pathway functioning independently from SAR. Another member of the Arabidopsis GLIP gene family GLIP2 has also been reported to affect defense responses through repression of the auxin signaling pathway. However, the transcript levels of GLIP2 were also enhanced by SA, methyl jasmonate (MeJA) and ethephon suggesting an involvement in multiple, even antagonistic signaling pathways. A GDSL lipase from hot pepper (CaGLIP1) with an organ and tissue specific expression pattern resulted in reduced symptoms of infection and repression of bacterial growth by virulent *Xanthomonas campestris* following silencing of the gene. Overexpression of CaGLIP1 caused enhanced disease susceptibility in transgenic Arabidopsis plants. Another GDSL lipase from pepper was highly inducible by MeJA as well as wounding and is also likely to modulate CaPR4 expression. Br–Si1, a putative lipase from chinese cabbage leaves and stems is highly inducible by the SA analog benzothiadiazole (BTH) and by *Pseudomonas syringae* infection, but neither by JA nor by ET. The GDSL lipase GER1 from rice can be induced by JA and red and far red light.

Class 3 lipases are also involved in plant stress responses. The family of alpha/beta-hydrolases containing class 3 lipase domains comprises three proteins with elemental roles in stress responses of Arabidopsis. These include PAD4, EDS1 and SAG101. They form a signaling system which functions as the main barrier against pathogens. Despite their biological characterization not much is known about the *in vivo* lipolytic activity and biological substrates of the class 3 lipases – it is actually uncertain whether they possess such an activity. The PRLIPs are also a group of class 3 lipases. Nine members of the gene family were identified in the Arabidopsis genome, three of them – PRLIP3, PRLIP8 and PRLIP9 – were reported to have orthologs in the rice genome. Expression levels of PRLIP1, PRLIP2 and PRLIP6 were highly induced by different biotic stress stimuli. In vitro esterase activity of the recombinant PRLIP1 enzyme was also confirmed experimentally. The exact biological role of the genes however is still unknown.

AIMS OF THE STUDY

The aim of the present study is to further characterize the plant PRLIPs, determinate their distribution among plants with available genome sequence information, and validate their differential expression in two model systems thale cress (*Arabbidopsis thaliana*) and grapevine (*Vitis vinifera*). In detail, the specific goals are:

To clarify sequence-level similarities among the class 3 lipases of Arabidopsis focusing on phylogenetic relationships between PRLIPs and other defense related members of the group.

To examine stress responses, and organ specific expression of the *PRLIP3, PRLIP9* and *PRLIP8* paralogs in *Arabidopsis thaliana*, which was not specified during the initial characterization of the gene family.

To reconstruct evolution of the *PRLIP* gene family in plants by screening sequenced plant genomes and determinate sequence-level similarities among the orthologs found.

To validate a functional differentiation within the gene family which was observed in Arabidopsis, by experimentally analyzing pathogen responses, and tissue specific expression of the grapevine *PRLIP* genes (VvPRLIPs).

MATERIALS AND METHODS

Microarray datamining, genomic database search and phylogenic analyses

Microarray data were retrieved from NBI GEO database (http://www.ncbi.nlm.nih.gov/geo/). Sequences of Arabidopsis class 3 lipases were identified via InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Databases of 25 sequenced and annotated plant genomes – available at Phytozome v7.0 Database (http://www.phytozome.net) – were screened to identify potential PRLIP homologs. Sequences were aligned with JalView program using ClustalW and MUSCLE methods, respectively. Phylogenetic analysis was carried out only on the satisfactory aligned region, using PAUP*4.0 computer program with the Distance option. A phylogenetic tree representing similarities of protein sequences was constructed with the neighbor–joining method. Bootstrap supports of 1000 replications are indicated.

Plant material and chemical treatments

The Columbia ecotype of *Arabidopsis thaliana* was used for the exeriments. Seedlings were transplanted to Jiffy pots after germination on soil. Plants were grown under controlled conditions. Vernalised cuttings of *Vitis vinifera* Cabernet Sauvignon and Pinot Noir cultivars were obtained from the Institute of Viticulture and Oenology, University of Pécs. Vine-stocks were rooted in perlite and the plants were allowed to grow under standard conditions. To induce resistance mechanisms, a fresh solution of sodium salicylate, benzothiadiazole, jasmonic acid and NaCl were applied as foliar spray or as soil drench. Ethylene treatments were carried out in an airtight container. Grapevine leaves infected with powdery mildew (*Erysiphe necator*) were collected in the vineyard. Grapevine leaves with different levels of disease were sorted, and pooled.

DNA/RNA isolation, reverse transcription and quantitative PCR

Genomic DNA was purified from leaves with the use of the DNeasy Plant Mini Kit. Total RNA was isolated from flash frozen plant tissues. After DNase I treatment, the quality of total RNA samples was checked by separating on a 1.2 % agarose gel containing 5 % formaldehyde. RNA quantities were measured spectrophotometrically in a NanoDrop ND–1000 spectrophotometer. cDNA was synthetized from 4µg (Arabidopsis) or 200 ng (grapevine) of total RNA using the First Strand cDNA Synthesis Kit with oligodT(18) primer following the manufacturer's protocol. Quantitative RT-PCR reactions were run in a reaction volume of 20 µl consisting of 10 µl Maxima^{TM} SYBRGreen/ROX qPCR Master Mix, 1.0 mM each of two primers, 8.5 µl water and 2 µl of cDNA

sample on a Step One™ Real-Time PCR System, with the following program: 40 cycles of 95 °C for 30 s; 60 °C for 30 s; and 72 °C for 60 s with an initial denaturation at 95 °C for 10 min. Gene expression levels were calculated by normalization relative to *Ubiquitin2* (Arabidopsis) or *Elongation factor 1–alpha* (*Vitis*) mRNA levels. Samples were measured in triplicates and relative quantification was done with the ΔΔCT method using the Step One™ 2.0 Software.

Cloning of PCR fragments

PCR products for cloning were generated with DreamTaq™ DNA Polymerase in a 23 µl reaction volume (2.3 µl DreamTaq™ Buffer, 0.5 mM dNTP mix, 1.0 µM of forward and reverse primers, 4 µl of gDNA or cDNA template 1.25 U DreamTaq™ DNA Polymerase and 12.5 µl water). PCR products were recovered from 1.5 % agarose gels with the GeneJET Gel Extraction Kit following the suppliers instructions. Amplicons were blunted and cloned to pJET1.2 vector using CloneJET PCR Cloning Kit. *Escherichia coli* DH5 alpha competent cells were transformed. The recombinant clones were analyzed by colony PCR with pJET1.2 forward and reverse primers according to the manufacturer's protocol. DNA samples were sequenced using Big Dye Terminator Cycle Sequencing Kit.

RESULTS

PRLIP proteins form a distinct group among Arabidopsis class 3 lipases

There are 9 PRLIP proteins in Arabidopsis each having a class 3 lipase domain. In a preliminary comprehensive sequence analysis these proteins formed a monophyletic group when compared to other 116 Arabidopsis protein sequences annotated as lipases, esterases and phospholipases. An unrooted phylogenetic tree was created with the neighbor-joining method which indicates a low level of homology, between the PRLIPs and other defense related class 3 lipases e.g. PAD4 and EDS1. However the monophyletic origin of the *PRLIP* gene family was further confirmed, and also supported by the characteristic exon-intron structure of the genes.

Differential expression pattern of the *PRLIP* genes

During our efforts to get gene expression information from public microarray databases about the Arabidopsis *PRLIP*s especially about the yet uncharacterised *PRLIP3*, *PRLIP9* and *PRLIP8* genes, we found many uncertainties. These include, even contradictional gene expression results, which might be due to bad quality gene prediction as well as to the high level of sequence similarity. To

validate the microarray data we used qRT-PCR technique and standardized chemical treatments to establish a comparative expression view among the yet unstudied *PRLIP* genes. In brief, *PRLIP3*, *PRLIP8* and *PRLIP9* showed a relatively high expression in all tissues tested with a maximum in root (*PRLIP3*), stem (*PRLIP8*) or siliques (*PRLIP9*). In contrast to, other family members these genes show rather a constitutive expression with only minor differences in plants under stress conditions.

Comparison between *PRLIP* clusters of two Arabidopsis species reveals rapid molecular evolution

Since the first characterization of the *PRLIP* gene family in the model plant *Arabidopsis thaliana*, the genome of *A. lyrata* has also been sequenced, so we compared the *PRLIP* gene cluster of these two closely related species. The constitutively expressed members *PRLIP3, PRLIP8* and *PRLIP9* are present in either of the genomes, located dispersedly in different regions, while the rest of the *PRLIP* genes encoding pathogen inducible members in *A. thaliana* occurred in one gene cluster in both species. The genes are in collinear position along the homolog chromosome segment, except for *PRLIP2* which is missing from the *A. lyrata* genome. There are additional significant disparities between the two species. In *A. lyrata* there is a short CDS between *PRLIP1* and *PRLIP4,* encoding a putative GTP-binding protein, which could not be located in *A. thaliana*. Another example for interspecific differences is the number of *PRLIP4* copies. Orthologs of the *PRLIP4* are in a collinear position in both regions compared, but there are two additional paralogs of this gene that are solely present in *A. lyrata*.

Plant PRLIPs can be divided to core and genome specific homology groups

Despite the close phylogenetic relatedness, 5 million years of independent evolution were efficient to develop remarkable disparity between *PRLIP* gene assortments of two Arabidopsis species. Therefore, we screened the proteome databases of 25 plants, to identify homologs of AtPRLIP proteins and to estimate sequence-level similarity among them. After critical revision of the BLAST matches, 135 sequences from 23 plant species were considered to be indisputable PRLIP homologs. The moss *Physcomitrella* possesses three PRLIP sequences which were used as outgroup in the analysis. The ingroup on the phylogenic tree consisted of Tracheophyte sequences. Accessions of *Selaginella* appeared as a sister in basal position, while Angiosperm sequences form two main clades, referred to as Clade I and Clade II. A variety of Eudicot sequences appear in the terminal branches in both of these clades: Clade I containing the homologs of AtPRLIP8, and Clade II containing the homologs of AtPRLIP3 and AtPRLIP9. In addition, four paralogs for PRLIP8 and

two for PRLIP3/9 are present in grasses, inferring a gene duplication event in one of the common ancestors. Perceptibly all of the sequenced plants have of at least one copy of PRLIP8 and PRLIP3/9, thus we term them core PRLIPs. Interestingly, the rest of the subclades in Clade II are mostly consisting of accessions from a single genome each. Unlike the core PRLIPs, these genome specific paralog groups contain proteins encoded in one gene cluster with the exception of subclades of *Mimulus* and *Glycine.* Altogether such genome specific subclades of PRLIPs could be established in 9 of the 23 analyzed species, and only one (including two *Medicago* accessions) is located within Clade I. Nevertheless there are unique PRLIP sequences – dissimilar to their core PRLIPs – in *Brachypodium, Manihot,* and *Citrus,* (one in each species, forming polyphyletic clades with *Oryza* accessions and each other respectively). The previously described and characterized *Arabidopsis thaliana* PRLIPs clustered on chromosome 5 (including PRLIP1, PRLIP2, PRLIP4, PRLIP5, PRLIP6 and PRLIP7) all belong to one of these specific paralog groups, and thus are singular in the Arabidopsis genus.

Core and genome specific *PRLIP* genes have different expression patterns in grapevine

Arabidopsis *PRLIP*s show a characteristic expression pattern depending on whether they belong to the core, or genome specific groups. In our further experiments, we analyzed the *PRLIP* gene assortment of grapevine to demonstrate this correlation between sequence-level similarities and functional differentiation in a distantly related plant species. As gene predictions of Genoscope grapevine genome database seemed to be poor for the *VvPRLIP*s, we used annotations of NCBI Map Viewer for the further analyses. A single copy for both core *PRLIP*s (designated as *VvPRLIP8* and *VvPRLIP3/9)* can be identified within the *Vitis* genome. In addition, a gene cluster containing duplicated genes of the *Vitis* specific *PRLIP*s is located on chromosome 16. Altogether, seven homolog gene loci in this region displayed homology to *AtPRLIP* genes (termed as *VvPRLIPE; VvPRLIPA; VvPRLIPF; VvPRLIPC; VvPRLIPG; VvPRLIPD* and *VvPRLIPB* to distinguish them from Arabidopsis specific *PRLIP* genes). The locus *VvPRLIPG* is apparently a gene fragment. Altogether, manual revision of the exon–intron junctions was needed to improve homology to other *VvPRLIP*s in case of *VvPRLIPC* and *VvPRLIPF*.

First, we identified genes that actually appear at transcript level. By exploiting the high level of sequence similarity among the clustered *VvPRLIP*s we designed a universal primer pair (VvUNIV), able to amplify all the predicted specific *VvPRLIP* loci. This capacity of the primers first was tested on *Vitis* genomic DNA. Sequence analyses confirmed that amplicons of all the seven homolog loci from the grapevine *PRLIP* cluster can be obtained. Next, *VvPRLIP* fragments from cDNA samples of both infected and uninfected grapevine leaves and all the other examined tissues

were amplified. These fragments were cloned and sequenced as described in Materials and Methods. At least 30 clones representing each sample were analyzed and our results indicated that only two paralogs *(VvPRLIPA* and *VvPRLIPC)* are detectable at mRNA level in the grapevine organs tested. Of note, it is possible that other members of the gene cluster are also transcribed in certain cell types or tissues, or under special conditions. Furthermore, a competition between the different templates for the universal primer might also occur favoring *VvPRLIPA* and *VvPRLIPC* transcripts.

To determine potential defense–related characteristics of the gene family we also studied the expression profiles of the *Vitis PRLIP* orthologs. In untreated grapevine plants (cultivar Pinot Noir) core *PRLIP*s display only small differences in expression among the compared tissues, with only 2.5-fold at the maximum. Conversely, specific *PRLIP*s, *VvPRLIPA* and *VvPRLIPC* showed remarkable level of expression in leaves compared to other organs.

We also studied expression changes of these genes in response to different stress regimen. Comparing *Vitis* leaves under different rates of powdery mildew infection revealed important changes in the mRNA levels of *VvPRLIPA* and *VvPRLIPC*. Low levels of infection caused 25-fold (Pinot Noir) or 80-fold (Cabernet Sauvignon) upregulation in *VvPRLIPA* transcript levels, while *VvPRLIPC* displayed 500-fold and more than 125-fold induction in the same infected leaves of the two cultivars, respectively. In contrast, a severe infection obviously decreased expression levels of grapevine specific *PRLIP* genes. As for the core *VvPRLIPs* only minor differences could be detected: *VvPRLIP8* showed 0.5-fold downregulation in moderately infected leaves of Pinot Noir, whereas *VvPRLIP3/9* displayed only a week induction with a maximum of 2-fold in both cultivars. We also analyzed vine stocks of the cultivar Pinot Noir treated with the stress inducers BTH and ET. Increasing expression of both *VvPRLIPA* and *VvPRLIPC* genes were observed in response to the SA analogue BTH treatment, while transcription of core *PRLIP*s was almost unaffected. ET treatment repressed transcription of all the *VvPRLIP*s especially *VvPRLIPA* to the yield of 0.1-fold after 48 hours and *VvPRLIPC* which was practically undetectable at transcript level after 2 days of inoculation.

DISCUSSION

The *PRLIP* family is a yet non-classified group of inducible defense related genes. Our findings suggest that several characteristics of them share similarities with other genes coding for different types of PR proteins.

First, members of the same *PR* family display extreme diversity in transcriptional inducibility. As discussed above, the *PRLIP* family of Arabidopsis and grapevine can also be divided into members closely related to plant pathogen interactions (the genome specific *PRLIP*s) and which are not (the core *PRLIP*s). The specific *PRLIP*s display high inducibility to biotic stress stimuli in both species. Interestingly, the Arabidopsis *PRLIP1* gene is upregulated by antagonistic signaling pathways, as it is the case for some members of rice *PR-1* family, especially *OsPR1a*. Another aspect of gene expression variation is the organ specific and developmentally regulated transcription in untreated plants. As we demonstrated *PRLIP* genes in healthy organs of Arabidopsis and grapevine including root-(*PRLIP6*, *PRLIP3*, *PRLIP4*) silique- (*PRLIP2*, *PRLIP9*) or leaf-(*PRLIP1*, *VvPRLIPA*, *VvPRLIPC*) specific members.

Another notable analogy between *PRLIP*s and some other PR families is the gene organization. We show that the genome specific *PRLIP* groups of different plants mostly occur in one single gene cluster each. Similarly, ortholog groups of tandemly repeated genes (displaying differential expression) encoding germin-ike proteins were also described from barley, as well as *PR*- genes of grapevine, from which 14 genes out of 21 form one cluster. Tandem arrays of *PR-1* genes are present in the Arabidopsis and *Oryza* genome including genes more homologous to each other than to other paralogs.

The third interesting common feature among *PRLIPs* and some other *PR* genes is the appearance of genome specific groups, occurred in different plant lineages. Rice or monocot specific groups of class III peroxidases were shown to be encoded by large numbers of paralog groups mostly located in gene clusters, similarly to taxon specific endoglucanases clustered in the Arabidopsis genome. Gene clusters encoding lipid transfer proteins were also proposed as being independently expanded in different plant phyla. Our analyses suggest that similar diversification of *PRLIP*s resulted in specific paralog groups conferring defense. It is also important to note that not all of the studied plant genomes posses an individual genome specific PRLIP group so this mechanism is likely to occur rather occasionally. However, this is not unusual as not all families of inducible defense-related proteins seem to be present in all plant species, for instance, Bowman-Birk type proteinase inhibitors can only be found in the *Poaceae* and *Fabaceae* families.

The emerging picture shows a novel family encoding inducible defense related proteins with regard to pathogen inducibility and differential basal gene expression. Together, our data provide important clues for further experiments especially for upcoming protein level characterization – essential to verify PR candidacy and physiological role of the gene family.

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