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Master Thesis

for the acquisition of the academic
degree Master of Science

Interaction of beetle polygalacturonases with putative plant inhibitory proteins

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Index

List of Figures

List of tables

1 Introduction

1.1 Plant Cell Wall and Plant Cell Wall Degrading Enzymes

Plant cell walls are as complex in their structural composition as in their functions. Not only do they play a role in the development, structural integrity and growth of the plant, but also control the sensing and signalling within cells as well as with the environment [1, 2]. Moreover, they also have to cope with and defend the plant against attacks and infections of herbivores (e.g. insects or nematodes) and pathogens (e.g. phytopathogenic fungi or bacteria). Plant cell walls differ between plant species [3] and can be influenced in their structure and composition by abiotic and biotic factors [4, 5].

Despite their differences, most plant cells walls are generally composed about 10 % of proteins and approximately 90 % of polysaccharides, such as cellulose, hemicellulose as well as pectic polysaccharides including homogalacturonan, rhamnogalacturonan II and substituted galacturonans (e.g. rhamnogalacturonan I, xylogalacturonan or apiogalacturonan [1, 2, 6-8]. These cell wall polysaccharides are the target for a variety of depolymerizing plant cell wall degrading enzymes (PCWDE), secreted by phytopathogenic microorganisms like fungi, bacteria, and nematodes to penetrate and colonize the plant. These include carbohydrate esterases (CE), glycoside hydrolases (GH) and polysaccharide lyases (PL) which break down the respective cell wall components [7, 9]. Out of these, pectinases, especially poylgalacturonases (PGs), are the first enzymes produced during a plant infection and are generally regarded as important pathogenicity factors for many plant pathogens such as the fungi *Botrytis cinerea* [10] and *Fusarium oxysporum* f. sp. *cubense* [11] or the bacterium *Ralstonia solanacearum* [12]. About ten percent of fungal species are able to cause diseases in plants [13] and the fast degradation of the cell wall leads to disruption and maceration plant tissue [14-16].

For a long time, it was thought, that the ability of successfully feeding on plants is restricted to microorganisms and for animals only possible due to symbiosis with microorganisms, which provide the necessary enzymes [17-19]. In 1998, the first gene encoding a functional cellulase in an insect, a termite, was discovered and identified and changed this assumption [20].

Besides phytopathogenic microorganisms, genes encoding for PCWDEs have since then been detected in in herbivorous insects [21-23] and nematodes [24]. Many studies indicate that these PCWDE-encoding genes have been transferred in the past from bacterial or fungal origins via horizontal gene transfer [19, 22, 25-27].

1.2 PCWDEs in *Phaedon cochleariae*

Of all herbivorous insects (Insecta), beetles (Coleoptera) of the Phytophaga clade (Chrysomeloidea and Curculionoidea) represent approximately half of the species [28]. More than 380 000 beetle species [29] [30] have been describes, of which of which 35% feed various kinds of plant material [31]. The horizontal gene transfer of PCWDEs to a beetle ancestor enabled the digestion of plant tissue and may have been the key to contribute to the diversification of plant-feeding beetles [25, 32].

The mustard leaf beetle *Phaedon cochleariae* is an insect pest species of the Chrysomelidae family. It feeds on Brassicaceae plants, and is often found in Europe [33] [34]. The screening of cDNAs from a gut library of *P. cochleariae* in 1999 detected a variety of PCWDEs including glucanases, β-glucosidases, pectinases and xylanases. [35]. More recently, several PCWDEs belonging to three different GH families could been identified through analysis of larval gut contents of *P. cochleariae*, by combining proteomics with transcriptome sequencing. Enzymes of the families GH11 (xylanases, EC 3.2.1.-), GH28 (PGs, EC 3.2.1.-), and GH45 (β-1,4-glucanases or cellulases, EC 3.2.1.-) were found [22, 23].

Of the GH 28 family, *P. cochleariae* possesses nine enzymes. Three of them are active as endo-PGs (GH28-1, -5, -9), which cleave the α-(1,4) linkages between D-galacturonic acid residues in homogalacturonan and degrade polygalacturonic acid into trimers, dimers and larger oligomers of galacturonic acid. GH28-4 is active as an oligogalacturonase, which hydrolyses the trimers formed by the endo-PGs. The remaining five (GH28-2, -3, -6, -7, -8) show no activity against any of the tested pectic substrates or other polysaccharides of the plant cell wall [19, 36] and their role remains unknown. The hypothesis about this role exists, that the inactive GH28 family members may act as „decoy" by binding inhibitory molecules to prevent the active PGs from inhibition of them [19].

1.3 Polygalacturonase-inhibiting proteins

Plants have developed various defence mechanisms against herbivore species as well as against phytopathogens. These range from physical barriers (e.g. thorns or waxy cuticular) to chemical defences (e.g. glucosinolates or cardiac glycosides).

One way to cope with PCWDEs is the production of proteininacious inhibitors, e.g. polygalacturonase-inhibiting proteins (PGIPs). These are extracellular plant proteins, which are bound to homogalacturonan [37] and inhibit PGs in their acitivity to digest pectin. The first gene of an PGIP was discovered in *Phaseolus vulgaris* in 1992 by Toubart et al. [28] and since then, many *pgip* genes and several PGIPs had been identified [38]. The complete or partial genes of a variety *pgip*s (or so-called *pgip*s due to similarities in the sequence without proven activity) from monocot and dicot plants can be found in databases nowadays [39, 40], but only a small number of them have been proven to truly encode for PG inhibiting proteins [16].
PGs as well as PGIPs belonging to large protein families, and their evolutionary development is assumed to be caused by an evolutionary arms race [41]. According to the large number of PGs, there is also a high variety of specific PGIPs to defend against them [16, 42]. Moreover, the number of PGIP-encoding genes differs from species to species, e.g. *Brassica napus* has got 16 of these genes [43]. *Arabidopsis thaliana* in contrast has only two of such genes [44], *Brassica rapa* ssp. *pekinensis* has got nine *pgip* (personal communication with Dr. Roy Kirsch).

They can be induced in the plants by various abiotic and biotic stresses such as cold, mechanical wounding, fungal infection or herbivory [16, 42, 43, 45, 46]. The induction cue for expressing the PGIP can vary among PGIPs [47, 48]. For example, the bean protein PvPGIP1 is upregulated only after wounding, PvPGIP2 is induced by wounding, oligogalacturonides and salicylic acid, PvPGIP3 only by oligogalacturonides and PvPGIP4 by none of these treatments [45]. Also for the PGIPs of *A. thaliana* the ways of inducing an effect in their regulation is different. Even if both were e.g. induced by wounding and Botrytis infection, the signal pathways are different for both. Whereas for the presence of exogenously added oligogalacturonides, known for being involved in wound response signalling, AtPGIP1 is induced, AtPGIP2 not [44].

The best studied PGIPs are the ones inhibiting fungal PGs, but there are also a few demonstrated examples for their inhibition against bacterial or insect PGs [49] [45, 50, 51]. However, there are no shown inhibitions of PGIPs against plants' own PGs.

Besides inhibition of PGs, the products of the degradation by endo-PGs, biological active oligogalacturonides are able to induce defence mechanisms of the plants, even in low concentration as endogenous elicitors [52-54].

For several fungal pathogens it was shown, that transgenic overexpression plants with PGIPs had significantly reduced symptoms of the diseases [55-57]. For ecample the overexpression of PvPGIP2 had a protective effect on tobacco plants, which were more resistant *P. parasitica* var. *nicotianae* then in comparison to wild type plants [58]. The role of PGIPs in relation to insect PGs remains currently elusive. That's why it was part of this master thesis, to assess the impact of putative PGIPs of *B. rapa* ssp. *pekinensis* on the beetle *P. cochleariae*.

1.4 PG-PGIP Interactions

PGIPs do not only vary in their numbers and expression patterns among different plant species, but also in their specificities and inhibitory activities towards PGs, when they originate from the same plant [47, 59]. Even a small variation like a single amino acid exchange can be significant for the function and the recognition ability of PGIPs [60]. Since PGIPs have leucine-rich repeat (LRR) structures, which provides the recognition ability of non-self-molecules due to protein-protein interactions [61-63]. The inhibition of the PGs by the PGIPs can occur in two ways there, competitive or non-competitive [64, 65].

There are several ways of showing inhibition or interaction of PGs with PGIPs in an experimental assay. Agarose diffusion tests for inhibition assays e.g. with positive results between PvPGIP2 and FmPG (later renamed FpPG), both PvPGIP2 and PvPGIP4 inhibit BcPG and both PvPGIP3 and PvPGIP4 inhibit the insect PGs of *Lygus rugulipennis* and *Adelphocoris lineolatus* [45, 66]. Chemical cross-linking tests are used to show interactions, here combined with small-angle X-ray scattering for a low-resolution structure of the formed complex between PvPGIP2 and FpPG [67].

Moreover, possible interactions can be assessed by using plasmon resonance, which successfully showed an interaction between VvPGIP1 and AnPGI. For the same pairing, colorimetric reducing sugar assays confirmed the interaction by showing an inhibition [68]. In most studies, no direct PG-PGIP interaction is demonstrated, but the inhibition of the enzyme acitivity of the PG. This could be shown directly in agarose diffusion tests or indirectly by expressing the PGIP in a transgenic plant to evaluate, if a higher resistance against e.g. fungal infections could be achieved, which is the case for ZmPGIP3 with PGs of *Rhizoctonia solani* [69]. In many of these studies, a relatively small variety of PGIPs is used, compared to the high number of known *pgip*-genes. Especially for PvPGIP2, many results are existing, which might be a sign, that its handling is easy relating to the stability after expression, what facilitates the research on and with it.

This possible advantage was also exploited in this master thesis by working with PvPGIP2 as part of a positive control. For putative PGIPS of *B. rapa* ssp. *pekinensis*, the handling seemed more problematic, since the proteins aggregate really fast which makes work with them or even their expression impossible. For this reason, the candidate proteins were expressed as GPI-anchored proteins on the surface of the membrane of Sf9 cells. In this way, the binding of the putative PGIPs to the cell wall was imitated like in natural environment and in addition, the problem of aggregation was avoided (personal communication with Wiebke Häger).

These GPI-achored proteins were used in this master thesis to establish a new interaction assay between PGIP and PG of various origin.

2 Aim of master thesis

In my master thesis I investigate the interaction of beetle polygalacturonases with putative plant inhibitory proteins. In a previous interaction study, eight proteins of *B. rapa* ssp. *pekinensis* were identified as interaction partners and possible inhibitors of the beetle enzymes PCO_GH28-1 and PCO_GH28-3 (master thesis Wiebke Häger).

For five of these eight candidates (Bra035741, Bra005917, Bra005917+9nt, Bra009238, Bra005916) and PvPGIP2 as a positive control for future assays, a transformation in the model plant *A. thaliana* should be performed by using the method of floral dipping [70]. The seeds should be selected for successful transformation [71] to grow a generation of heterozygous plants containing the candidate genes, laying the foundation to create homozygous overexpression lines.

For the remaining three candidates (Bra005919, Bra038700, Bra034774), already previously created homozygous *A. thaliana* overexpression lines should be used for feeding experiments with *P. cochleariae.* The weight gain of feeding larvae and their development should be monitored to study the effect of the candidate proteins on *P. cochleariae*, which are expressed by the overexpression lines in comparison to wild type *A. thaliana* plants (Col-0).

Furthermore, a novel interaction assay between PGIPs and PGs should be established to circumvent former aggregation problems of PGIPs with this new method. This interaction assay should be established using the well-studied PvPGIP2-FpPG system [45, 67]. The applicability of the method should be tested with AnPGII, confirming a previously published inhibition of AnPGII by PvPGIP2 [45].

The novel method should then be used to elucidate the interaction of various combinations of PvPGIP2 and Bra005919 with PGs of fungal and beetle origin.

3 Material and Methods

3.1 Material

3.1.1 Plants

A. thaliana	Reared at Max Planck Institute for Chemical Ecology

3.1.2 Cells

Escherichia coli	
One Shot® TOP10 Competent Cells	Thermo Fischer Scientific GmbH, Bonn
Agrobacterium tumefaciens (GV3101)	Provided by Wiebke Häger
Sf9 cells	Provided by Wiebke Häger

3.1.3 Enzymes/Proteins

Pfu Turbo Cx Hotstart Polymerase	Carl Roth GmbH + Co. KG, Karlsruhe
USER enzyme mix	New England Biolabs GmbH, Ipswich, MA, USA
T4 Ligase	New England Biolabs GmbH, Ipswich, MA, USA
FpPG	Provided by Wiebke Häger, expressed by *Pichia pastoris*
PvPGIP2	Provided by Wiebke Häger, expressed by *Pichia pastoris*
Phospholipase C Protein, Phosphatidylinositol-Specific	Thermo Fischer Scientific GmbH, Bonn
AnPGII	Provided by Wiebke Häger, expressed by *Pichia pastoris*

3.1.4 Kits

DNA Clean & Concentrator-5 Kit	Zymo Research Corporation, Irvine, CA, USA
GeneJET Plasmid Miniprep Kit	Thermo Fischer Scientific GmbH, Bonn
PureLink® HiPure Plasmid Filter Midiprep Kit	Thermo Fischer Scientific GmbH, Bonn
SuperSignal™ West Dura Extended Duration Substrate Kit	Thermo Fischer Scientific GmbH, Bonn

Taq PCR Master Mix Kit	Qiagen GmbH, Hilden
Innuprep RNA Mini Kit	Analytik Jena AG, Jena

3.1.5 Consumable Material

Amersham Hyperfilm DCL	GE Healthcare Life Sciences, München
Amicon Ultra- 15 10K Centrifugal Devices	Merck KGaA, Darmstadt
CELLSTAR® CELLreactor™ Filter Tubes 15 ml and 50 ml	Greiner Bio-One GmbH, Frickenhausen
Criterion™ XT Bis-Tris Precast Gels	Bio-Rad Laboratories GmbH, München
Extra Thick Blot Filter Paper, Precut, 7.5x10 cm	Bio-Rad Laboratories GmbH, München
Gene Pulser®/Micropulser™ electroporation cuvettes, 0.2 cm	Bio-Rad Laboratories GmbH, München
Immun Blot PVDF Membrane	Bio-Rad Laboratories GmbH, München
Microplate Nunc™ 0.2 ml flat bottom 96-well	Thermo Fischer Scientific GmbH, Bonn
Polypropylene Columns 1 ml / 5 ml	Qiagen GmbH, Hilden

3.1.6 Chemicals

2-(N-morpholino)ethanesulphonic acid (MES)	Carl Roth GmbH + Co. KG, Karlsruhe
Acetic acid	Carl Roth GmbH + Co. KG, Karlsruhe
Agar	Sigma-Aldrich Chemie GmbH, Hamburg
Anti-myc-HRP Antibody	Thermo Fischer Scientific GmbH, Bonn
Anti-V5-HRP Antibody	Thermo Fischer Scientific GmbH, Bonn
Biotin	Sigma-Aldrich Chemie GmbH, Hamburg
BSA	Bio-Rad Laboratories GmbH, München
DL-Dithiothreitol	Sigma-Aldrich Chemie GmbH, Hamburg
dNTPs	Carl Roth GmbH + Co. KG, Karlsruhe
Ethanol	VWR International GmbH, Darmstadt
FuGENE® HD Transfection Reagent	Promega Corp, Fitchburg, WI, USA
GBX Developer and Replenisher	Kodak GmbH, Stuttgart
GBX Fixer and Replenisher	Kodak GmbH, Stuttgart

Gentamicin	Thermo Fischer Scientific GmbH, Bonn
Glycerol	Carl Roth GmbH + Co. KG, Karlsruhe
HisPure ™ Cobalt Resin	Thermo Fischer Scientific GmbH, Bonn
Hydrochloric acid	Thermo Fischer Scientific GmbH, Bonn
Imidazole	Carl Roth GmbH + Co. KG, Karlsruhe
Isopropanol	Carl Roth GmbH + Co. KG, Karlsruhe
Kanamycin	Merck KGaA, Darmstadt
LiChrosolv® Water	Merck KGaA, Darmstadt
Methanol	Carl Roth GmbH + Co. KG, Karlsruhe
Murashige and Skoog Basal Salt Mixture (MS)	Merck KGaA, Darmstadt
O'Gene Ruler 1 kb DNA Ladder Plus	Thermo Fischer Scientific GmbH, Bonn
Orange DNA Loading Dye 6x	Thermo Fischer Scientific GmbH, Bonn
PageBlue Protein Staining Solution	Thermo Fischer Scientific GmbH, Bonn
PageRuler Plus Prestained Protein Ladder	Thermo Fischer Scientific GmbH, Bonn
Polygalacturonic acid demethylated, prepared from citrus pectin	Megazyme International Ireland, Bray, IRL
Protease Inhibitor Cocktail (for plant cell and tissue extracts)	Sigma-Aldrich Chemie GmbH, Hamburg
Powdered milk	Carl Roth GmbH + Co. KG, Karlsruhe
Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories GmbH, München
Rifampicin	Merck KGaA, Darmstadt
Ruthenium Red	Sigma-Aldrich Chemie GmbH, Hamburg
SeaKem® LE Agarose	Lonza Verviers, S.p.r.l, Verviers, B
Silwet-L-77	Thermo Fischer Scientific GmbH, Bonn
Sodium acetate	Carl Roth GmbH + Co. KG, Karlsruhe
Sodium chloride	Carl Roth GmbH + Co. KG, Karlsruhe
Sodium dodecyl sulphate	Carl Roth GmbH + Co. KG, Karlsruhe
Sucrose	Carl Roth GmbH + Co. KG, Karlsruhe
SYBR® Safe DNA Gel Stain Thermo	Thermo Fischer Scientific GmbH, Bonn
Tris(hydroxymethyl)aminomethane (Tris)	Carl Roth GmbH + Co. KG, Karlsruhe
Tryptone	Duchefa Biochemie B.V, Haarlem, NL
Tween-20	Sigma-Aldrich Chemie GmbH, Hamburg
XT Reducing Agent (20x)	Bio-Rad Laboratories GmbH, München
Yeast extract	Duchefa Biochemie B.V, Haarlem, NL
Yeast Nitrogen Base (YNB)	Thermo Fischer Scientific GmbH, Bonn

3.1.7 Primer

Bra035741_UF	ggcttaauATGAAGCTCAACGTCTTCGTATCAC
Bra005917_UF	ggcttaauATGGGTAAGACAACGATACTGCTC
Bra009238_UF	ggcttaauATGAGTAAGGCAACGACACTGC
Bra005916_UF	ggcttaauATGGATAAGATAACGACTACATTGCTC
PvPGIP2_UF	ggcttaauATGTCCTCAAGCTTAAGCATAATTTTGG
URP_myc_His-STOP_UR	ggtttaauATGATGATGATGATGATGGTCGACG
Seq_F:	TCACTCATTAGGCACCCCAGG
Seq_F2:	CTTCGCTATTACGCCAGCTGG
Bra035741_Int_Seq	CGCCGGAGATGAGGTTGTTAC
Bra005917_Int_Seq	CTCTTTTAAATGATCCAAATGACTCTGG
Bra009238_Int_Seq	AACGACCTAAATAACTCTGGTATTGG
Bra005916_Int_Seq	CTCCAAATGACTCTGGTATTGAACC
PvPGIP2_Int_Seq	TAGGAGTCGGGGATGGCG

3.1.8 Plasmids

pIB/V5-His TOPO® Vector — Thermo Fischer Scientific GmbH, Bonn

Provided by Dr. Roy Kirsch

pIB/V5-His-*PCO_GH28-1* (codes for PG PCO_GH28-1 ORF from *P. cochleariae*)

pIB/V5-His- *PCO_GH28-2* (codes for PG PCO_GH28-2 ORF from *P. cochleariae*)

pIB/V5-His-*PCO_GH28-3* (codes for PG PCO_GH28-3 ORF from *P. cochleariae*)

pIB/V5-His- *PCO_GH28-4* (codes for PG PCO_GH28-4 ORF from *P. cochleariae*)

pIB/V5-His-*PCO_GH28-5* (codes for PG PCO_GH28-5 ORF from *P. cochleariae*)

pIB/V5-His- *PCO_GH28-6* (codes for PG PCO_GH28-6 ORF from *P. cochleariae*)

pIB/V5-His-*PCO_GH28-8* (codes for PG PCO_GH28-8 ORF from *P. cochleariae*)

pIB/V5-His- *PCO_GH28-9* (codes for PG PCO_GH28-9 ORF from *P. cochleariae*)

pPICZα A vector — Provided by Wiebke Häger

pPICZα A- Bra035741 (codes for Bra035741 ORF from *B. rapa* ssp. *pekinensis*)

pPICZα A- Bra005917 (codes for Bra005917 ORF from *B. rapa* ssp. *pekinensis*)

pPICZα A- Bra005917+9nt (codes for Bra005917+9nt ORF from *B. rapa* ssp. *pekinensis*)

pPICZα A- Bra009238 (codes for Bra009238 ORF from *B. rapa* ssp. *pekinensis*)

pPICZα A- Bra005916 (codes for Bra005916 ORF from *B. rapa* ssp. *pekinensis*)

pPICZα A- PvPGIP2 (codes for PvPGIP2 ORF from *Phaseolus vulgaris*)

pCAMBIA230035SU (digested with PacI and Nt.BbvCI)

3.1.9 Devices

Biophotometer	Eppendorf AG, Hamburg
E. coli Pulser™ Transformation Apparatus	Bio-Rad Laboratories GmbH, München
GeneGenius Gel Imaging System	Biocon, India
Infinite® M200	Tecan Group Ltd. Männedorf, CH
Mastercycler EP Gradient	Eppendorf AG, Hamburg
NanoDrop ND-1000 Spectrophotometer	Peqlab Biotechnolgie GmbH, Erlangen
Tissue Lyser II	Qiagen GmbH, Hilden
Trans-Blot® transfer cell	Bio-Rad Laboratories GmbH, München

3.1.10 Software

Edit Seq	DNASTAR, Inc., Madison, WI, USA
i-control™ Microplate Reader Software	Tecan Group Ltd. Männedorf, CH
Microsoft Excel® 2010	Microsoft Corporation, Redmond, WA, USA
PCR Primer Design Tool Eurofins Genomics	http://www.mwg-biotech.com/, MWG-Biotech AG, Ebersberg
SeqMan Pro	DNASTAR, Inc., Madison, WI, USA

3.1.11 Buffer and Media

XT MES Running Buffer	Bio-Rad Laboratories GmbH, München
TBS 10x	Bio-Rad Laboratories GmbH, München
10x Pfu Turbo Buffer Cx	Agilent Technologies Inc., Santa Clara, CA, USA
10x Cut Smart Buffer	New England Biolabs GmbH, Ipswich, MA, USA
10x T4 DNA Ligase Buffer	New England Biolabs GmbH, Ipswich, MA, USA
Sf-900™ II SFM	Thermo Fischer Scientific GmbH, Bonn
Rotiphorese® 50x TAE Buffer	Carl Roth GmbH + Co. KG, Karlsruhe
S.O.C. medium	Thermo Fischer Scientific GmbH, Bonn
10x Tris/Glycin Buffer	Bio-Rad Laboratories GmbH, München
XT Sample Buffer (4x)	Bio-Rad Laboratories GmbH, München

BMMY Medium	1%	Yeast extract
	2%	Tryptone
	0.1 M	Potassium phosphate buffer, pH 6.0
	1.34%	YNB
	$4 \cdot 10^{-5}$%	Biotin
	1%	Methanol
IMAC Binding Buffer	50 mM	Sodium phosphate buffer, pH 7.4 / pH 8.0
	0.5 M	NaCl
IMAC Wash Buffer	50 mM	Sodium phosphate buffer, pH 7.4 pH 8.0
	0.3 M	NaCl
	10 mM	Imidazole
IMAC Elution Buffer	50 mM	Sodium phosphate buffer, pH 7.4
	0.3 M	Imidazole
Low Salt LB Agar	1%	Tryptone
	5%	Sodium chloride
	5%	Yeast extract
	2%	Agar
Low Salt LB Medium	1%	Tryptone
	5%	Sodium chloride
	5%	Yeast extract
MS Agar plates (15)	1.35 g	MS Salt (0.5x)
	0.3 g	MES (2.5 mM)
	3.6 g	agar
	600 ml	H_2O
		KOH for pH 5.8
Hypotonic Buffer	20 mM	Tris-HCl pH 7.5
	5 mM	EDTA
	1 mM	DTT
Sucrose Buffer	20 mM	Tris-HCl pH 7.5
	5 mM	EDTA
	1 mM	DTT
	500 mM	sucrose

gDNA extraction buffer	200 mM	Tris-HCl pH 7.5
	250 mM	NaCl
	25 mM	EDTA
	0.5%	SDS
Staining Solution	50 %	Methanol
	7 %	Acetic Acid
	0.1 %	PageBlue Protein Staining Solution
Destaining Solution	50 %	Methanol
	7 %	Acetic Acid
Transfer Buffer	10%	Methanol
	1 x	TG Puffer

All buffers and media were prepared using ddH_2O or ultrapure H_2O. For all other reactions LiChrosolv® Water was used.

3.2 Methods

3.2.1. Transformation of *A. thaliana*

3.2.1.1 PCR

The open reading frames of the five candidates (Bra035741, Bra005917, Bra005917+9nt, Bra009238, Bra005916) and PvPGIP2 were provided in pPICZαA vectors by Wiebke Häger (sequences in Supplementary Data 10). The respective sequences are fused with a myc and His_6 tag.

Both, forward (Bra035741_UF, Bra005917_UF, Bra009238_UF, Bra005916_UF, PvPGIP2_UF, see full sequences in Material 3.1) and reverse primer (URP_myc_His-STOP_UR, see full sequence in Material 3.1) were designed to introduce a USER cassette for subsequent USER cloning [72]. The melting temperatures were determined with the PCR Primer Design Tool of Eurofins Genomics [genomics, #2429]. The PCR was performed according to the Pfu Turbo Cx instruction manual [Agilent, #2430] with a Mastercycler ep Gradient.

	Volume [µl]
Primer forward (10 µM)	1
Primer reverse (10 µM)	1
dNTPs (10 mM per dNTP)	1
10x Pfu Turbo Buffer Cx	1.5
Pfu Turbo Cx Hotstart Polymerase	1
DNA template (100 ng)	X
H_2O	44.5 - X

Temperature	Time	
95 °C	2 min	
95 °C	30 s	30 x
55 °C	30 s	
72 °C	2 min	
4 °C	∞	

Table 1: Volumes used for a 50 µl PCR (left) and PCR program (right) with gene specific primers.

3.2.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to determine PCR amplification success. Therefor, 5 µl of the samples mixed with 1 µl loading buffer (6x Orange DNA Loading Dye) and 4 µl of the DNA Ladder (O' Gene Ruler DNA Ladder Mix) was applied onto an 1.2 % agarose gel with 0.005 % GelGreen and run for 30 min at 120 V. The gels were documented with a Gene Genius Bio Imaging System.

3.2.1.3 Purification of PCR products

PCR products were purified with the DNA Clean & Concentrator™-5''Kit according to the manufacturer's instructions [73]. All centrifugation steps were performed at 12 000 x g. The PCR samples were mixed with five volumes of DNA binding buffer, transferred to a Zymo-Spin™ Column in a collection tube and centrifuged for 30 s. After discarding the flow-through, 200 µl of DNA wash buffer was added to the column and again centrifuged for 30 s. After discarding the flow-through, the washing step was repeated. After transferring the column into a new collection tube, 10 µl elution buffer was applied onto the column and incubated at room temperature for 2 minutes. Finally, the column was centrifuged for 1 min to collect the purified DNA. The concentration and quality were measured with a Nanodrop ND-1000 Spectrophotometer.

3.2.1.4 USER Cloning

The USER cloning was performed as described by Nour-Eldin et al. [72]. In this method, the used primers contain a single uracil, which is cut out of the forward strand with the USER enzyme mix. In this way, sticky ends are formed at the end of the PCR products, which could be easily combined with the digested vector afterwards. The PCR product was incubated with the USER enzyme mix for at least 20 min at 37 °C.

	Volume [µl]
PCR product (1.5-2 µg)	10
USER enzyme mix	2
10x Cut Smart Buffer	4
H_2O	24

Table 2: Volumes used for 40 µl USER cloning.

Afterwards the samples were purified with the DNA Clean & Concentrator™ kit as described in 3.2.1.3 ***Purification of PCR products*** and the DNA concentration and quality were measured with the Nanodrop ND-1000 Spectrophotometer.

The PCR products were mixed with the vector pCAMBIA230035SU, which was digested with PacI and Nt.BbvCI beforehand, and T4 ligase and incubated for 30 min at room temperature.

Then the temperature was increased to 60 °C for 10 min to stop the reaction by heat inactivation.

	Volume [µl]
purified PCR product (45 ng)	X
vector pCAMBIA230035SU (90 ng)	Y
10x T4 DNA Ligase Buffer	1
T4 Ligase	1
H_2O	10 – X – Y

Table 3: Volumes used for a 10 µl ligation reaction.

3.2.1.5 Transformation of *E. coli*

5 µl from each ligation reaction were mixed with 25 µl of *E. coli* One Shot® TOP10 Competent Cells and placed on ice for 15 min. After a heat shock with 42 °C for 30 s, 250 µl of S.O.C-medium was added immediately, and the mixture was incubated 1h at 37 °C and 250 rpm.

The *E. coli* cells were plated on LB Agar plates (kanamycin 50 µg/ml) and incubated overnight at 37 °C.

3.2.1.6 *E. coli* colony PCR

Single colonies of each construct were picked the next day with toothpicks and dipped into tubes with colony PCR reaction mixture. Afterwards they were used to inoculate 50 µl LB medium (kanamycin 50µg/ml) and cultivated (37 °C, 250 rpm). For the colony PCR a Mastercycler ep Gradient was used and amplification success was monitored by agarose gel electrophoresis as described in 3.2.1.2 ***Agarose gel electrophoresis***.

	Volume [µl]
Taq PCR Master Mix	10
Primer forward (10 µM)	0.5
Primer reverse (10 µM)	0.5
H_2O	9

	Temperature	Time
	94 °C	10 min
35 x	94 °C	20 s
	55 °C	20 s
	74 °C	2 min
	4 °C	∞

Table 4: Volumes for a 20 µl colony PCR (left) and PCR program (right) with Taq PCR Master Mix Kit and gene specific primers.

For all positive samples, the LB medium cultures were transferred to 15 ml Falcon tubes with 5 ml LB medium (kanamycin 50 µg/ml) and cultivated overnight (37 °C, 250 rpm).

3.2.1.7 Miniprep of plasmids from *E. coli* cultures

Isolation of plasmids from *E. coli* was performed with the Gene JET Plasmid Miniprep Kit according to the manufacturer's instructions [74]. 4 ml of each cell culture was centrifuged for 2 min at 6 800 x g and the supernatant was discarded. After resuspension of the cell in 250 µl of the Resuspension Solution, 250 µl of the Lysis Solution were added and the tubes were inverted several times to get a homogeneous mixture. Then, 350 µl of the Neutralization Solution were mixed to the solution by inverting the tubes several times again, followed by a centrifugation step for 5 min at 16 000 x g to pellet the cell debris and chromosomal DNA. Afterwards, the supernatant was pipetted to the Gene JET spin column and centrifuged again for 1 min at 12 000 x g and the flow-through was discarded. The column was then washed two times with 500 µl of the Wash Solution (1 min of centrifugation at 16 000 x g) and the flow-through was discarded both times. An additional centrifugation (1 min at 16 000 x g) was carried out to remove residual Wash Solution. After transfer of the column to a new 1.5 ml microcentrifuge tube, the plasmid DNA was eluted with 50 µl of the Elution Buffer after 2 min incubation at room temperature by a final centrifugation step (2 min at 12 000 x g). The concentration and quality were measured with the Nanodrop ND-1000 Spectrophotometer.

3.2.1.8 Verification of inserts by sequencing

To confirm the correct sequences of the inserts, sequencing was carried out by our in-house sequencing service.

	volume [µl]
DNA (130 ng)	X
Primer (10 µM)	0.5
H_2O	6 - X

Table 5: Volumes for a 6 µl sequencing approach with gene specific primers.

Each construct was sequenced with three different primers, two reverse primers (F and F2) and an internal primer (primer sequences see Material 3.1). The sequences were verified with the software SeqMan Pro.

3.2.1.9 Midiprep of plasmids from *E. coli* cultures

After the confirmation of the correct sequences, one of each *E. coli* clones inoculated in 100 ml LB medium (kanamycin 50 µg/ml) and were grown overnight (37 °C, 250 rpm). The midiprep of the overnight cultures was carried out with the PureLink® HiPure Plasmid Filter DNA Purification Kit according to the manufacturer's instructions [75]. The cultures were centrifuged for 5 min at 6 800 x g. Meanwhile, 15 ml of Equilibration Buffer (EQ1) was applied onto a column with inner filtration cartridge and drained by gravity flow. After centrifugation, the supernatant was discarded and the pellet was resuspended in 10 ml Resuspension Buffer (R3) with RNAse A, mixed with 10 ml Lysis Buffer (L7) and incubated for 5 min. Afterwards, 10 ml of the Precipitation Buffer (N3) were added and mixed by inverting the tube. The mixture was then applied onto the column. After draining of the liquid, the filtration cartridge was discarded, and the column was washed with 20 ml Wash Buffer (W8). The purified DNA was eluted with 5 ml of the Elution Buffer (E4). The DNA in the eluate was precipitated with 3.5 ml isopropanol (30 min at 4°C) and pelleted at 16 000 x g (30 min at 4 °C). After discarding the supernatant, the pellet was washed with 3 ml of 70 % ethanol and subsequent centrifugated (10 min, 16 000 x g, 4°C). The supernatant was removed, and the pellet was air-dried for at least 10 min before it was resuspended in 100 µl H_2O. Then the concentration and quality of the purified plasmids were measured with the Nanodrop ND-1000 Spectrophotometer.

3.2.1.10 Preparation of electrocompetent *Agrobacterium tumefaciens*

Agrobacterium tumefaciens were inoculated from a -80 °C stock in 5 ml LB-medium with 25 µg/ml gentamicin and 150 µg/ml rifampicin in a 50 ml cell culture tube with a filter cap. Since rifampicin is a light-sensitive antibiotic, all following steps were carried out with as little light as possible. Gentamicin and rifampicin were added to the culture medium, since the used *A. tumefaciens* cells include two accessory plasmids for an increase in virulence for the plant infection. For this reason, these two antibiotics were used for selection. The cultures were cultivated overnight in the dark (28 °C, 250 rpm). After measuring the OD_{600}, the cells were pelleted for 5 min at 5000 x g and washed with 10 ml water. To remove all salts for the electroporation transformation, the wash step was repeated four times. Then, the cell concentration was adapted to 1 x 10^{10} cells/ml. Aliquots of 100 µl were frozen in liquid nitrogen and stored at -80 °C.

3.2.1.11 Electroporation of *A. tumefaciens*

For the transformation of the *A. tumefaciens* with the purified plasmid DNA, 2 µg DNA in 4 µl H_2O of each construct was mixed with one 100 µl aliquot of the *A. tumefaciens*. This mixture was transferred to a pre-cooled electroporation cuvette (0.2 cm) on ice and pulsed two times with 2400 V in an E. coli Pulser^{TM} Transformation Apparatus. Immediately after pulsing, 500 µl of S.O.C-medium were added and the mixture was cultivated for 4h (28 °C, 150 rpm).
The transformed pCAMBIA230035SU plasmid carries a kanamycin resistance. The *A. tumefaciens* were plated on LB agar with kanamycin (50µg/ml), gentamicin (25 µg/ml) and rifampicin (150 µg/ml) and incubated for two days at 28 °C.

3.2.1.12 *A. tumefaciens* colony PCR

Single colonies of each construct were picked and inoculated into 5 ml of LB medium (kanamycin 50µg/ml, gentamicin 25 µg/ml, rifampicin 150 µg/ml) and cultured overnight (28 °C, 250 rpm). Since the cell wall of *A. tumefaciens* is more stable than of *E. coli*, the colonies couldn't be used directly for the colony PCR, because the DNA would be way too less. For this reason, they were cultured to get more cells, 1 ml of the Agrobacteria cultures were centrifuged with 5 000 x g for 5 min, the supernatant has been removed and the pellet was resuspended in 50 µl H_2O. After that, the suspension was heated for 10 min at 95 °C and centrifuged again for 5 min with 5 000 x g. The supernatant was then used to measure the concentration of the DNA. To verify the correct integration of the plasmids in *A. tumefacien*s, the colony PCR was carried out with 200 - 400 ng as described in 3.2.1.6 **E. coli *colony PCR***.
Out of the positive cultures, for each construct one culture was chosen for the further steps.

3.2.1.13 Glycerol stocks of *E. coli* and *A. tumefaciens*

To store *E. coli* and *A. tumefaciens* for future repetitions of experiments, the respective clones were inoculated in 5 ml LB medium (*E. coli*: kanamycin 50 µg/ml, *A. tumefaciens*: kanamycin 50µg/ml, gentamicin 25 µg/ml, rifampicin 150 µg/ml) and cultured overnight (*E. coli*: 37 °C, 250 rpm, *A. tumefaciens:* 28 °C, 250 rpm). For each

construct 4 ml of the overnight culture were centrifuged for 5 min at 5 000 x g. After the supernatant was removed, the pellet was resuspended in 1 ml of the overnight culture together with 500 µl of sterile 50% glycerol. This mixture was then frozen in liquid nitrogen in a cryo tube and stored at -80 °C.

3.2.1.14Floral Dip

The transformation of *A. thaliana* was carried out according to the Floral Dip method described by Bernhardt et. al [Bernhardt, 2012 #6].

A. tumefaciens infects plant tissues and integrated the genetic material in the plant genome. To stably transfer this to the next generation, it has to integrate in the seeds. Thus, for each construct, five plants of six week old flowering *A. thaliana* from long-day climate chamber (6 hours dark, 18 h light) were used for the transformation. Already formed seed capsules or completely opened flowers were removed beforehand.

For the *A. tumefaciens* pre-culture, 5 ml LB medium (kanamycin 50µg/ml, gentamicin 25 µg/ml, rifampicin 150 µg/ml) were inoculated from the -80 °C stocks. These cultures were cultivated overnight (28 °C, 150rpm) and then transferred into 300 ml new LB medium (kanamycin 50µg/ml, gentamicin 25 µg/ml, rifampicin 150 µg/ml) and again grown overnight (28 °C, 150rpm).

The *A. tumefaciens* cultures were harvested by centrifugation for 13 min at 3200 x g. The supernatant was discarded, and the pellets were resuspended in 100 ml of 5 % sucrose solution. Then the OD_{600} of the individual solutions was measured and adjusted to an OD_{600} of 0.8 in approximately 500 ml 5 % sucrose solution. Shortly before dipping, 0.02 % Silwet-L-77 was added to the sucrose- *A. tumefaciens* solutions of each construct as an adhesive and wetting agent, which helps to infect the plants efficiently. The plants were inverted, and the stalks and flowers were dipped into the solution for 1 min with gentle movement. Then it was placed horizontally on wet cloths in a tray for overnight. For each construct, a separate tray was used and before new plants were dipped in the sucrose solution of a new construct, the whole equipment and workspace was disinfected to avoid co-infection of several constructs in one plant. The next day, the plants were brought to an upright position and were transferred to the long day climate chamber. When the seeds had developed on all plants, the watering was stopped, and the stalks were coated in paper bags to avoid a transfer of seeds of the

different constructs. The dried seeds, seven weeks after the floral dip, were then sieved to get rid of the capsules of the seeds, other plant material and dust.

3.2.1.15 Selection of seeds

Successfully integrated plasmid in the plant genome confer kanamycin resistance. For the selection of the successfully transformed seeds, they were spread on MS agar plates (kanamycin 50µg/ml) in a sterile environment. To avoid any contamination, the seeds were sterilised by washing them twice with 70 % ethanol for 10 min and then once with 100 % ethanol for 5 min. They were dried on sterile filter paper in separate petri dishes to avoid cross-contamination and spread over the plates equally. As negative and positive control, wildtype (Col-0) seeds and transformed seeds expressing GFP were plated on MS agar (kanamycin 50µg/ml), respectively. The plates were sealed with parafilm and were stored in the dark at 4 °C for two days for stratification. Afterwards, they were exposed to 6h of light in a climate chamber at 20 °C, to get the light stimulus for the synchronized germination of the seeds, followed by two days of darkness, where the growth continued due to hypocotyl extension. In the subsequent two days in permanent light, the successfully transformed seeds with kanamycin resistance grow photoautotrophically by the accumulation of chlorophyll, whereas the negative ones, which lack to grow normally due to the missing kanamycin resistance, appear yellow and smaller. The positive plants, which looked healthy and possess green leaves and stems, were transferred to a long day climate chamber for 20 days to increase plant size and form roots, which are large enough to support the plants in the soil. The surviving positive plants were transferred from the agar plates into pots with soil by our gardening service, where they grew under long day climate conditions until they flowered and produced seeds again.

3.2.1.16 Extraction and PCR of gDNA from *A. thaliana*

To verify the inserts in the selected *A. thaliana*, gDNA was extracted from the leaf tissues of all plants. For this purpose, the lid of a 1.5 ml reaction tube with three metal beads was used to punch two discs out of young leaf tissue and frozen in liquid nitrogen. The tubes were put in the Tissue Lyser II at 50 Hz for 1 min to homogenize the leaf material and were centrifuged for 1 min afterwards. This and all following centrifugation steps were carried out at 16 000 x g. Then the samples were mixed with

400 µl of gDNA extraction buffer and centrifuged for 1 min again to pellet the debris. 300 µl of the resulting supernatant were transferred to a new tube, mixed with an equal volume of isopropanol and incubated for 5 min, followed by 5 min of centrifugation to pellet the DNA. The supernatant of each tube was discarded, and the pellets dried for approximately 20 min at room temperature. The pellet was resuspended in 100 µl of H_2O and the DNA concentration was measured with the Nanodrop ND-1000 Spectrophotometer. Afterwards a colony PCR was carried out with 100 ng as described in 3.2.1.6 **E. coli *colony PCR***.

For all plants with negative colony PCR, the PCR and/or extraction was repeated, to double check the results. The plants, which were still negative for an insert were discarded.

3.2.2 Feeding assays with homozygous transformed *A. thaliana* plants

3.2.2.1 Pre-experimental feeding assay

To assess the optimal number of larvae per plant for the main feeding assay, four day old *P. cochleariae* larvae were placed on five weeks old wild type *A. thaliana* plants. In duplicates, 0, 2, 3, 5, 10 or 20 larvae were placed on the plants and the amount of consumed plant material was checked after ten days of feeding. To avoid movement of larvae between the plants, transparent plastic cups with removed bottom were put over the plants at an early growth state and the openings covered with elastic cloth.

3.2.2.2 Feeding assay

For the assessment of the effect of putative PGIPs on *P. cochleariae*, neonate larvae were put on plants of three overexpression lines (Bra005919, Bra038700, Bra034774) of *A. thaliana* and their growth and development was compared with larvae feeding on wild type plants. The three *A. thaliana* overexpression lines were used in the third generation to assure homozygosity and were provided by Wiebke Häger. For the main feeding experiment, *A. thaliana* was grown in transparent plastic cups, as described in 3.2.2.1 ***Pre-experimental feeding assay***and 30 six weeks old plants of similar size from a short day climate chamber (8 h light, 16 hours dark) were used for each line. On each of the plants, three neonate *P. cochleariae* larvae were placed. The plants were placed randomly on three trays in a climate chamber (21 °C, 50 % humidity, 10 h light (0.5h 50

%, 9h 100 %, 0.5h 50% light intensity). On day 11 the larvae were collected and weighted. During the weighing period of approximately six hours, the larvae were placed in 6-well plates (one well per plant) with a leaf of the respective *A. thaliana* line. Three larvae of six plants per *A. thaliana* overexpression lines and wild type, respectively, were dissected by Dr. Roy Kirsch. The three larval samples per plant were pooled. The gut tissue was homogenized in Lysis Solution RL (innuPREP RNA Mini Kit) for subsequent RNA isolation and gene expression analysis and the gut content put in 50 mM citrate-phosphate buffer for further enzyme activity assays. The larvae and plant material were frozen in liquid nitrogen and stored at -20 and -80 °C, respectively, for further analysis.

The larvae of the remaining 24 plants per line were put back on the plants. After approximately 16 days, the larvae move into the soil to pupate. When no more larvae could be seen on the plants, the plants were cut above ground to easily monitor the hatching of the beetles. To lure the beetles out of the soil when they hatched, a leaf of Chinese cabbage was put in the cups instead, which was replaced daily. To record the day of hatching, the cups were checked every day at the same time and all hatched beetles were removed. When no more beetles hatched for at least three days, the monitoring was ended. The statistical analysis (Shapiro-Wilk, Kruskal-Wallis) of the larval weight gain was performed with the software SigmaPlot.

The feeding assay was repeated once. The second time, larvae were weighed on day 11 without dissection, thus all larvae were put back on the plants, increasing sample size for the development monitoring.

3.2.2.3 RNA extraction of gut tissue

For further analyses to check what happened with *P. cochleariae* during the feeding experiment, the RNA of the gut tissue was extracted with the innuPREP RNA Mini Kit according to the manufacturer's instructions [76]. All six taken samples of each mutant were used. Each of the tubes contained the tissue of three larvae which feed on the same plant. The tissue was grinded and homogenized by the metal beads by vortexing after 450 µl of Lysis Solution RL was added. A centrifugation was carried out for 1 min with 16 000 x g. Afterwards, the supernatant was transferred to the Spin Filter D, which was placed in a new Receiver Tube. It was then centrifuged again for 2 min with 10 000 x g for the binding of DNA on the filter. The filtrate, which still contained the DNA was

then mixed with the equal volume (400 µl) of 70 % ethanol and this mixture was applied to the Spin Filter R, which was placed on a new Receiver Tube before. To bind the RNA on this filter, it was centrifuged for 2 min with 10 000 x g. Afterwards, the filter was washed two times, first with 500 µl Washing Solution HS, then, after centrifuge for 1 min with 10 000 x g and removing the flow-through, 700 ml of Washing Solution LS were used, followed by the same centrifugation step. The filtrate was discarded, the Spin Filter R with the Receiver Tube were centrifuged again for 2 min with 16 000 x g to get rid of remaining rests of the Washing Solution. The filter was transferred to a new tube, 89 µl Elution Buffer were added and it was incubated for 2 min. Then a centrifugation was carried out for 1.5 min with 11 000 x g. The eluate contained the desired RNA of the gut tissue. Afterwards, the samples were digested, to get rid of potentially remaining DNA. To each eluate, 10 µl Turbo DNA Buffer (10x) and 1 µl Turbo DNAse were added and the digestion was carried out at 37 °C for 30 min. The samples then were purified with RNA Clean & Concentrator™-5 according to the instructions of the manufacturer [77]. All centrifugation steps for this were carried out with 16 000 x g. For this each sample with its 100 µl volume were mixed with two volumes of RNA Binding Buffer and an equal total volume (300 µl) of 100 % ethanol. This mixture was applied to the column in a collection tube and centrifuged for 30 s. The flow-through was discarded and after 400 µl of RNA Prep Buffer were added, the centrifugation was repeated and the flow-through was removed again. A washing step was performed with 700 µl RNA Wash Buffer and a centrifugation for 30 s. After discarding the flow-through, 400 µl RNA Wash Buffer were applied and the samples were centrifuged for 2 min, to avoid any remaining wash buffer in the column. The column was transferred to a new tube and 25 µl DNase/RNase-Free Water were added to the column matrix and centrifuged for another 30 s to eluate the RNA out of it. The concentration of the purified RNA was measured with the Nanodrop ND-1000 Spectrophotometer afterwards.

3.2.3 Interaction assay between PGs and putative inhibitory proteins

3.2.3.1 Membrane preparation of Sf9 cells

Stable Sf9 cell lines expressing BraPGIP3_GPI and PvPGIP2_GPI were established and provided by Wiebke Häger. These proteins are membrane-anchored to the outside of the

cell surface by a GPI anchor to prevent aggregation, a common problem, when working with LRR proteins (personal communication with Wiebke Häger). Plasma membranes of the Sf9 cells were isolated by differential centrifugation. The adherent Sf9 cells were scraped from the culture flasks and resuspended in the culture medium by pipetting. The cells were pelleted by centrifugation for 10 min at 500 x g and 4 °C and washed with 3 ml PBS per culture flask. After that, the cells were resuspended in 2.5 ml hypotonic buffer per culture flask and placed on ice for 20 min, to let the cells swell and burst. For further lysis of the cells, they were ground in a dounce homogenizer with a tight pestle. After checking the cells under microscope to see, if at least 70-80 % of the cells were lyzed, an equal volume of sucrose buffer, and again a centrifugation was carried out for 10 min with 1 200 x g at 4 °C to pellet the cell nuclei. The supernatant homogenised as described above. The pooled supernatants were centrifuged for 15 min with 10 000 x g at 4°C to pellet the plasma membrane with the attached membrane proteins. The pellet was washed with citrate-phosphate buffer (40 mM, pH 5.0), followed by centrifugation for 5 min with 12 000 x g at 4 °C. This pellet was resuspended until a homogenised solution formed by adding citrate-phosphate buffer (40 mM, pH 5.0, with protease inhibitor). Aliquots of the membrane were made and were stored at -20 °C.
This membrane preparation was carried out for Sf9-cells with the GPI-anchored membrane proteins PvPGIP2 and BraPGIP3 as well as wildtype Sf9-cells as a control for future assays.

3.2.3.2 Purification of PGs expressed in yeast

The PGs FpPG and AnPGII were expressed in *Pichia pastoris* and provided by Wiebke Häger. For the purification of the secreted, His_6-tagged proteins from the culture medium, it was mixed with an equal volume of IMAC buffer. To enable that the beads to bind as many proteins as possible, it was incubated for 1h at 4 °C on a roll mixer, then poured in 5 ml Polypropylene Column, which retains the beads. After draining of the liquid by gravity flow, the beads were washed with IMAC buffer (at least 20 times the column volume). To elute the proteins, one column volume of elution buffer was applied to the column. The imidazole in the elution buffer replaces the His_6-tagged proteins from the column. After draining, the elution fraction was collected and applied again to increase the protein concentration per fraction. It was then incubated for 5 min on the column, drained, collected and applied to the column again. After another 5 min

incubation, it drained and was collected as elution fraction 0 - 2. Afterwards again one column volume was added to the column and the steps were repeated to collect elution fraction 3 - 4 together. Then again one column volume was applied to the column, incubated for 5 min and collected as elution fraction 5. The protein concentration of these elution fractions was determined by Bradford assay.

3.2.3.3 Concentration and buffer exchange of purified proteins

To concentrate protein solutions, Amicon Centrifugal Filter Devices were used according to the manufacturer's instructions for all yeast-expressed enzymes for the interaction assay [78]. The samples (up to 15 ml) were applied to the filter device and centrifuged at 4 000 x g for 10 min. By washing the sample repeatedly with H_2O, a buffer exchange into H_2O was achieved. The sample was concentrated from a volume from up to 15 ml to 200 µl and stored at 4 °C (FpPG) or -20 °C (AnPGII). A Bradford assay was carried out to determine the protein concentration.

3.2.3.4 Protein quantification by Bradford Assay

The Bradford assay is a method for the determination of protein concentrations by determining a colour change depending on the concentration. A high absorbance can be measured, when a high protein concentration is in the sample, since the Coomassie® Brilliant Blue G-250 dye, which is contained in an acidic solution in the Bradford reagent, got a shift in the absorbance maximum from 465 nm to 595 nm when proteins are bound. The samples are compared with standard concentrations of BSA between 0 and 350 ng/µl to determine their protein concentration.

A 96-well-plate was used for the measurement, the wells filled with the samples or when necessary, diluted with H_2O to have a total volume of 20 µl always. Afterwards 200 µl of Bradford reagent (1:5 with H_2O) were added to all wells and mixed, to minimize a temporal difference between the wells. The mixture was incubated at room temperature for 10 min and the absorbance at 595 nm was measured with the Tecan Infinite® M200 plate reader.

3.2.3.5 Transfection of Sf9 cells with *P. cochleariae* PGs

For the expression of *P. cochleariae* GH28 family members (PCO_GH28-1, -2, -3, -4, -5, -6, -8, -9) in Sf9 cells, pIB/V5 vectors containing the respective ORFs were provided by Dr. Roy Kirsch. 6-well plates with 70 % confluent Sf9-cells were used. For each construct, 2.4 µg of the plasmids were mixed with fresh culture medium to a total volume of 92.8 µl and 7.2 µl of FuGENE were added. The mixture was incubated at room temperature for 10 min. During this time, the culture medium of the 6-well plates was removed and 1 900 µl fresh medium was applied carefully. To each well, 100 µl of the FuGENE-plasmid mixture was added drop by drop at several points of the well. Afterwards the plates were slightly shaken horizontally and incubated until were confluent. Then, the culture medium containing the secreted proteins was removed and stored at 4°C until further use.

3.2.3.6 Agarose diffusion tests as activity assay of PGs

To test the activity of the expressed PGs, an agarose diffusion assay was used. The plates were prepared at least one day before usage.

	Volume [ml]
polygalacturonis acid (1 % in H_2O, w/v)	2
citrate phosphate buffer (0.2M)	5
agarose (1 % in H_2O, w/v)	8
H_2O	5

Table 6: Volumes for a 20 µl ADT plate.

Small holes in equal distances were punched out by using cut pipette tips. The samples were pipetted into the holes in two 5 µl steps. The plates were incubated for 2 h or overnight at 40 °C. After that, the plated were stained with 25 ml ruthenium red (0.1% w/v in H_2O) for 1 h. Afterwards the plate was washed with H_2O for at least 1 h to remove unbound ruthenium red. When the polygalacturonic acid in the plates was digested during the time in the oven at 40 °C by the added PGs, that diffused in the gel, the ruthenium red couldn't bind the long-chained polymers on these spots anymore. That's why the regions, where the polygalacturonic acid was digested, seemed colourless after staining, while the undigested regions looked orange-pink, coloured by the ruthenium red. The regions appeared as circles, because the mixture, which was tested, was added into the punched holes and the liquid was spreading circular during

the ingress and digested the the polygalacturonic acid circular, when an activity had existed.

3.2.3.7 SDS-Page and Western Blot

For the detection of proteins SDS-PAGE and Western Blotting was used. First, the SDS-PAGE was carried out to separate the proteins along their molecular weight. Therefore, the samples were mixed with 2.5 µl 4x XT Sample Buffer, 2.5 µl SDS (10 %) and 0.5 µl 20x XT Reducing Agent and applied to the gel pockets. The volume of sample was depending on the size of the gel and in this way with the pocket size and the concentration of protein in the sample. To check for the molecular weight of the detected proteins afterwards, 5µl of PageRuler™ Prestained Protein Ladder was used as size standard. The SDS-Page was run in 1x XT MES Running Buffer for 80 min with 125 V. For the following Western Blot, the PVDF-membrane was activated in methanol for 5 min to become less hydrophobic, after that, the membrane and the gel were inserted in transfer buffer for 5 min, the Extra Thick Blot Filter Paper for some seconds. For the transfer of the proteins from gel to membrane, a Trans-Blot® transfer cell was used, and the Blotting has run for 30 min with 100 V in Western Blot Transfer Buffer. Afterwards the membrane was blocked with milk solution (5 % milk powder in TBS-T) to block unspecific binding sites for 60 min. Then the solution was changed, so the specific antibody for the proteins, which had to be detected, was diluted by milk solution (5 % milk powder in TBS-T) according to the type of antibody (Anti-V5-HRP Antibody: 1:20000, Anti-myc-HRP-Antibody: 1:1000). The incubation with the antibody was carried out shaking overnight and then the membrane was washed three times with TBS-T for 5 min and once with TBS for 5 min. For the visualisation of the proteins, the Super Signal™ West Dura Extended Duration Substrate Kit was used according to the instructions of the manufacturer [79]. In a dark room, the membrane was covered with an Amersham Hyperfilm DCL chemiluminescence film for different periods of time, developed and fixed with GBX Developer and Replenisher and GBX Fixer and Replenisher solution. Afterwards the membrane was stained by a staining solution for at least 30 min, decolorized with a destaining solution for at least 30 min and air-dried.

3.2.3.8 Establishment of an Interaction Assay

Due to problems with aggregation of LRR-proteins and only short times of stability in experiments of the past, stable Sf9 cell lines expressing BraPGIP3_GPI (Bra005919), one of the candidates of B. *rapa* ssp. *pekinensis*, and PvPGIP2_GPI were established and provided by Wiebke Häger. These are membrane-anchored proteins, which are located at the outside of the cell surface connected by a GPI anchor and in this way the contact between them is reduced and the aggregation prevented or at least minimized (personal communication with Wiebke Häger). In the natural environment, PGIPs also appear as bound molecules connected to pectin [11], not as soluble proteins, what is mimicked by this method.

For an assessment of their ability to interact with several PGs or proteins of the same gene family, an interaction assay had to be established. For the establishment, the combination of PvPGIP2_GPI and FpPG was chosen again as positive control, due to their already shown interaction [10].

After it was successfully achieved to reproduce the results of the interaction between PvPGIP2_GPI and FpPG, the established method was transferred to use it for the putative PGIP BraPGIP3_GPI of B. *rapa* ssp. *pekinensis* and several PGs.

In this way, the method was changed step by step until an interaction between PvPGIP and FpPG could be seen with clear results.

As starting point of the establishment of the interaction assay, 244 µg of membrane of wildtype (WT) and the same amount of GPI-anchored PvPGIP2 membrane out of the membrane preparation were used. These amounts each equalled four interaction pairings in the following assay. Both had to be washed first. Therefore, a centrifugation was carried out for 5 min with 10 000 x g at 4 °C, the supernatant was discarded and the pellet, which contained the membrane including the bound proteins, was resuspended in 500 µl sodium acetate buffer (100 mM, pH 4.6). The solution was centrifuged with the same conditions again, the supernatant was again removed and after resuspension, this washing step was repeated once again. The resulting pellet was then resuspended in 100 ml of the sodium acetate buffer. At the same time, a BSA sample (2 µg/µl) as control and a purified sample of FpPG in elution buffer (25 ng/µl) were centrifuged for 10 min with 16 000 x g and the supernatant was used to make dilutions of both to achieve samples with a concentration of 0.05 ng/µl of the proteins in sodium acetate buffer. Then, mixtures of 25 µl of the membrane samples (61 µg membrane) with 1 µl of these

proteins (0.5 ng) were created according to the following scheme. BSA was used as protein control instead of the enzyme FpPG and the wildtype membrane was used as control for the membrane with the GPI-anchored PvPGIP. Furthermore, controls without membrane or without interaction partners for the membrane were tested. Sodium acetate buffers were used in these cases to provide the same volumes in the samples.

membrane	interaction partner
25 µl PvPGIP	1 µl FpPG
25 µl PvPGIP	1 µl BSA
25 µl PvPGIP	1 µl sodium acetate buffer
25 µl WT	1 µl FpPG
25 µl WT	1 µl BSA
25 µl WT	1 µl sodium acetate buffer
25 µl sodium acetate buffer	1 µl FpPG
25 µl sodium acetate buffer	1 µl BSA

Table 7: Sample scheme for interaction assay (first version)

The samples were all incubated for 1.5 h in the test tube rotator and afterwards a centrifugation was carried out for 30 min with 10 000 x g at 4 °C. Since all (putative) PGIPs which were used in the interaction assays were myc-tagged and all PGs were V5-tagged, SDS-Page and Western Blot of two gels incubated with different antibodies had to be carried out. For this reason, two times 10 µl of the supernatant was taken from each of the samples and mixed with sample buffer for the SDS-Page. The pellets which still contained the membranes including the membrane proteins were washed by resuspending them in 500 µl of sodium acetate buffer, centrifuge them for 10 min with 10 000 x g at 4 °C and discarding the supernatant. This step has been repeated for another two times and then the pellet was resuspended in 26 µl of sodium acetate buffer and again two times 10 µl were taken and mixed with the same sample buffer. All supernatant samples as well as membrane samples with the sample buffer were boiled for 5 min at 95 °C and applied to the gels. The SDS-Page and the Western Blot were carried out as described before. After the first version of working with the membrane proteins and trying to show an interaction by this assay, several changes in the procedure of the interaction assay were performed step by step and were checked with Western Blots every time. After the first approach, 228 µg membrane instead of 244 µg were used for the following steps and the enzyme FpPG was purified to have a higher

protein concentration and less impact of the elution buffer on the possible interaction. Then several concentrations of FpPG were tested to get the optimal concentration for a clear band on the Western Blot. Afterwards all assays were carried out with 5 ng FpPG per sample. Furthermore, the possible binding of membrane proteins and the interaction partners should have been stabilized, by trying to use cross-linking with formaldehyde (FA) [6], with a final concentration of 1 % in the mixed sample. The membrane samples mixed with the interaction partner (FpPG/BSA) were added up with buffer (sodium acetate buffer) to a volume of 30 µl, incubated for 1 h and then, 10 µl of 4% FA or buffer (as negative control) were added and incubated overnight at room temperature. In further assays, it was tested to incubate membrane and interaction partner for 1h at 4 °C and instead of incubating with FA overnight at room temperature, to incubate also for 1h at 4 °C and later in another approach the incubation time with FA was extended to overnight with 4 °C. Moreover, another way of interaction was tested by using phospholipase C to cut the GPI anchor before incubating the membrane with a possible interaction partner. Therefore, the PvPGIP membrane, after washing, was incubated for 1 h with different concentrations of phospholipase C or buffer (as negative control) at room temperature or 4 °C and with or without boiling the samples for 5 min at 95 °C afterwards before applying the samples to the gel. This was tested without putative interaction partner but nevertheless, the supernatant as well as the membrane (pellet) fraction were tested in Western Blots to check for the success of separating the membrane proteins from the membrane by cutting the GPI-anchor and to check for possible aggregation of the membrane proteins which would be visible in the Western Blot. Afterwards the usage of phospholipase C was tested again in connection with an interaction partner, but this time only the supernatant fraction was used. This means that the membranes were incubated with 0.2 U of phospholipase C for 1 h at room temperature, followed by an earlier centrifugation step for 30 min with 10 000 x g to separate membrane and former membrane-bound proteins and afterwards an 1h incubation between supernatant (still 25 µl for each sample) and interaction partner at 4 °C. Then the FA or buffer (as control) was added and incubated for 1 h, later overnight at 4 °C and finally at 16 °C overnight, all without boiling the samples before applying them on the SDS-Page. From that approach, all following steps in the establishment of the interaction assay, used low-binding tubes to avoid interaction or cross-linking of any parts of the mixture with the tubes itself, especially during overnight incubation and used 226 µg for four samples of membrane at the beginning of the assay, so 56.5 µg per

sample. Additionally, instead of using the test tube rotator, heat blocks with shaking feature were used to provide more proximity between membrane proteins and possible interaction partners without losing parts of the sample e.g. in the lids of the tubes. Furthermore, all uses of sodium acetate buffer in the assay, for washing as well as for resuspending and dilution, were replaced by using citrate phosphate buffer (40 mM, pH 5.0) since this buffer is anyway used for the membrane preparation.

According to this, the finally established interaction assay contained the washing steps of the membranes, their cutting by the usage of phospholipase C for 1 h at room temperature, to separate the GPI-anchored membrane proteins from the membranes themselves. After the centrifugation for 30 min with 10 000 x g, the volume of the supernatant, which equals 56.5 µg of the former membrane was incubated with the possible interaction partner for 1 h at 4 °C followed by the incubation with FA (final concentration 1 %) for cross-linking membrane proteins with PGs as potential interaction partners at 16 °C overnight. All samples including the different controls (with wildtype membrane, without any membrane, without PG, without FA) were applied to SDS-Page, without boiling them before, and the Western Blots were checked afterwards incubated by anti-myc and anti-V5 antibodies.

The established procedure of the interaction assay was used to test different pairings of potential interaction partners. As membrane proteins, PvPGIP and BraPGIP3 (and the wildtype membrane) were tested and as interaction partners the purified PGs FpPG and AnPGII, both yeast-expressed by Wiebke Häger were chosen, after they were already tested as not purified samples in the culture medium of the yeasts. Moreover, the PGs PCO_GH28-1, PCO_GH28-2, PCO_GH28-3, PCO_GH28-4, PCO_GH28-5, PCO_GH28-6, PCO_GH28-8 and PCO_GH28-9 were incubated in all combinations with the membrane proteins. They were expressed in Sf9-cells and were used as not purified samples in Sf9-medium, after testing the impact of the culture mediums of yeast or Sf9-cells on the interaction assay. All interaction assays, which showed positive results between membrane proteins and PGs were repeated at least once.

4 Results

4.1 Transformation of plants

In a previous interaction assay, several putative inhibitors of *P. cochleariae* PGs were identified from *B. rapa* ssp. *pekinensis* cell wall protein extracts [80]. Five out of eight candidates (Bra035741, Bra005917, Bra005917+9nt, Bra009238, Bra005916) as well as PvPGIP2 (as positive control for future assays) were cloned and used to transform *A. tumefaciens*. All tested *A. tumefaciens* clones were positive for their respective insert in the colony PCR after transformation and selection (Supplementary Data, figure 17).

One *A. tumefaciens* clone was used to transform *A. thaliana* plants using the Floral Dip method [70], respectively. The plasmids containing the ORFs as well as a kanamycin resistance gene integrate into the *A. thaliana* genome. Thus, the seeds were selected on MS agar with kanamycin.

Successfully transformed seeds germinated and formed healthy green leaves, while the plants from untransformed seeds were small, with colourless or light-yellow leaves and died after a few days. This is exemplarily shown for Bra005917 + 9nt (Figure 1, A). These phenotypes were confirmed by plating wildtype (Col-0) seeds without any transformed plasmids as a negative control (Figure 1, B) and seeds from transformed, homozygous GFP lines (Figure 1, C) as positive control. For every construct, more than 50 successfully transformed plants were observed per two selection plates out of around 2000 seeds. Of these, approximately 35 healthy plants were transferred into pots with soil, respectively, as soon as they had formed several leaves and root of sufficient length to provide enough nutrients and water for the plants (Figure 1, D). They were grown until they produced seeds for the next plant generation.

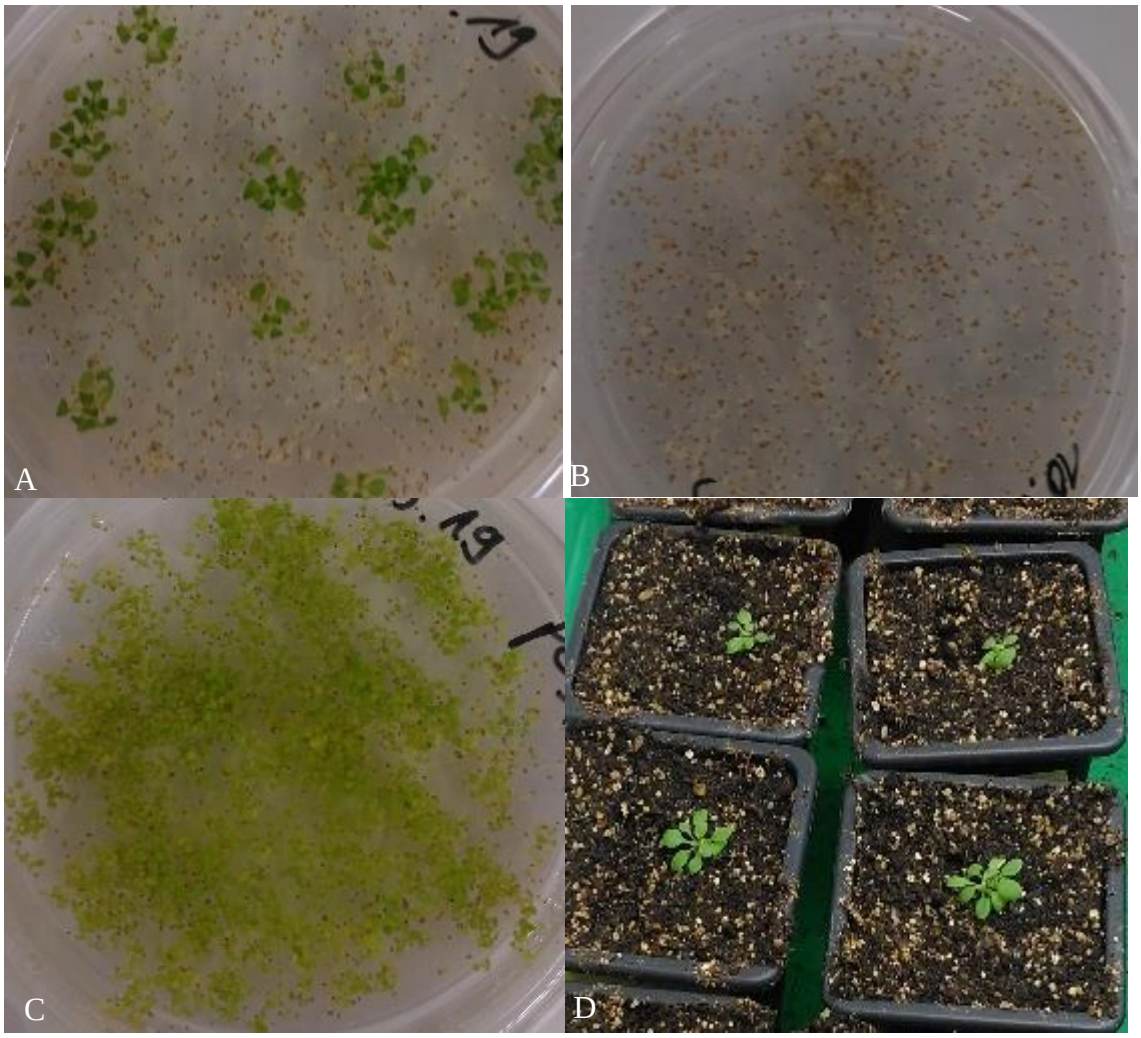

Figure 1: Selection of *A. thalina* seeds. *A. thaliana* seeds from floral dip plants were plated onto MS agar with kanamycin (50 mg/ml). Successfully transformed plants are green, whereas not transformed plants have pale yellow leaves and die after a few days. A: seeds from *A. thaliana* floral dip plants, exemplarily shown for Bra005917 + 9nt after 3 weeks; B: wildtype (Col-0) seeds (negative control) after 3 weeks; C: seeds from transformed, homozygous GFP line (positive control) after 3 weeks; D: positive plants after transfer to soil after 4 weeks.

The gDNA was extracted from the leaf tissue, respectively, and used to verify the inserts by PCR. An agarose gel of the plant screening is exemplarily shown for Bra009238 in Figure 2. When no PCR product was visible, the gDNA extraction and PCR was repeated to exclude false negatives due to the extraction process. These showed positive results for most repeated plants (data not shown). The plants that were negative in the PCR were discarded.

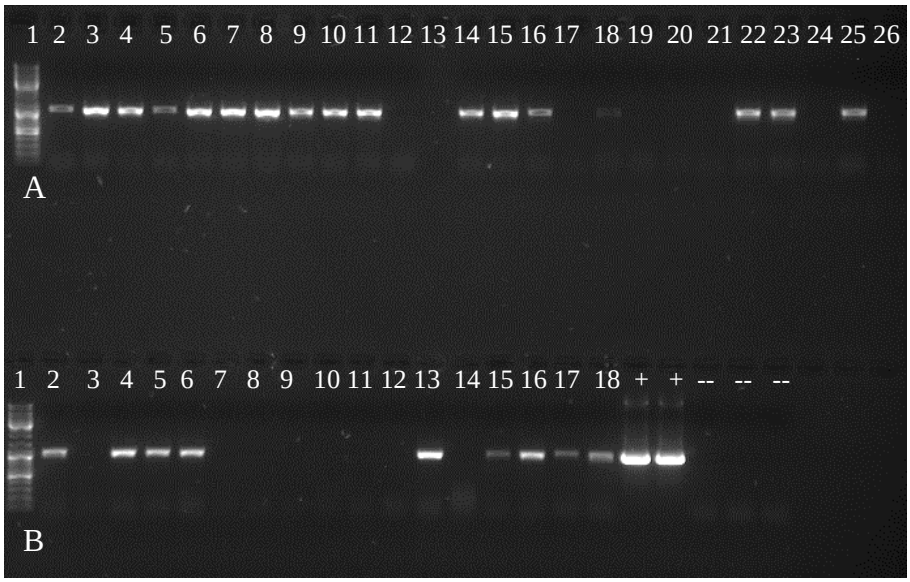

Figure 2: Agarose gel of gDNA screening of Bra009238 *A. thaliana*. The gDNA was extracted from A. thaliana leaves and used for PCR with gene-specific primers. Positive controls (+) were amplified from the respective pCAMBIA2300_35SU plasmid. Negative controls (--) used gDNA from *A. thaliana* GFP lines. A: 2-26: PCR product from gDNA of *A. thaliana* plants 1-25; B: 2-18: PCR product from gDNA of *A. thaliana* plants 26-42. A1, B1: GeneRuler DNA Ladder Mix.

All in all, more than 90% of the tested *A. thaliana* plants were positively tested for the insert by PCR and can be used to create homozygous overexpression lines in the future.

4.2 Feeding Assay

The feeding assay was used to assess the effect of putative inhibitory proteins expressed by the transformed feeding plants on the beetle *P. cochleariae.* Besides wild type plants as reference, overexpression lines of A. thaliana (Bra005919, Bra038700, Bra034774) were used. They were provided as homozygous transformed plants by Wiebke Häger. Since the way from transformation of wildtype plants with the candidate genes until homozygous plants expressing the respective proteins lasts several generations and the time period of the master thesis was limited, the feeding assay had to be done with the provided plants of the remaining candidates.

A pre-experiment revealed three larvae per plant as optimum for the main feeding assay. The number was chosen as high as possible to offer as much replicates as possible per plant while providing the larvae food *ad libidum* during their development time between neonate stadium and pupae. In the main feeding assay, three neonates of P. cochleariae were placed on 30 plants per overexpression line (Bra005919, Bra038700, Bra034774) and also on 30 wild type plants as reference.

The weight gain as well as the development of the larvae until hatched beetles was monitored. As verification of the results, the experiment was carried out two times. In the first approach 18 beetles per plant construct were dissected for further analyses with the gut tissue and gut content and the empty plants were cut, frozen in liquid nitrogen and stored at -20 °C.

The larvae of *P. cochleariae* were weighed after 11 days of feeding. The weight of the individuals varied between 1.1 and 15.6 mg in the first (Figure 3A) and 0.98 and 14.8 mg in the second experiment (Figure 3 B), respectively. A high variation of the larval weights could be seen for all larvae, including those feeding on the wildtype plants. For both approaches, no statistically significant differences in the weight of larvae feeding on wildtype plants compared to ones feeding on the overexpression lines could be detected. One-way ANOVA and one-way ANOVA on ranks (p=0,05) was used for normally distributed and not normally distributed data, respectively. Even though no significant differences were detected, there seems to be a slight trend. It seemed like the larvae feeding on plants transformed with Bra034774 were a little lower in weight than the ones feeding on wild type and the larvae feeding on overexpression lines with Bra038700 and Bra005919 had a little more weight than the ones on the wild type plants.

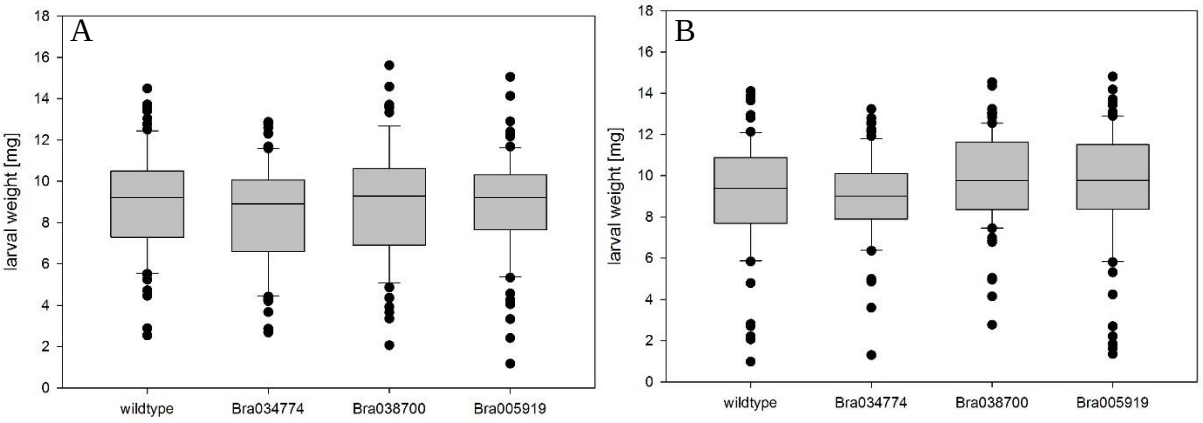

Figure 3: Larval weight of *P. cochleariae* after feeding on *A. thaliana* overexpression lines and wild type. Neonate P. cochleariae larvae were placed on A. thaliana overexpression lines (candidates Bra034774, Bra038700, Bra005919) and wild type plants and the weight gain was monitored after 11 days. A and B represent two independent experiments. No significant differences were detected between larvae feeding on the overexpression lines compared to the wild type. One-way ANOVA for normally distributed data and one-way ANOVA on ranks not normally distributed data was used for Shapiro-Wilk and Kruskal-Wallis test respectively, both with p=0,05.

Additionally, the further development of *P. cochleariae* was monitored by daily counting the hatching of adult beetles. In both experiments, the beetles started to hatch after about 22 days over a period of approximately one week. The hatching ended three days later in the repetition than in the first experiment. The total number of hatched beetles was smaller for the first approach than for the second one, because no larvae were dissected during the repetition. For the remaining larvae in both, the survival rate was in similar range, 47-55% and 48-60%, respectively.

For both experiments, the hatching curves of the beetles were similar between *P. cochleariae* feeding on wild type as well as *A. thaliana* overexpression lines (candidates Bra034774, Bra038700, Bra005919) (Figure 4).

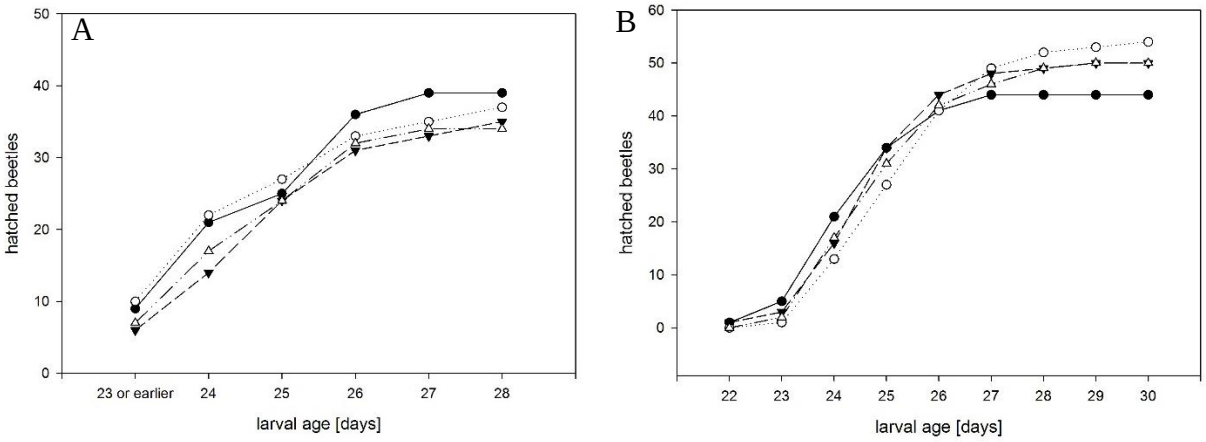

Figure 4: Development of *P. cochleariae* after feeding on *A. thaliana* overexpression lines and wild type according larval age. Neonate P. cochleariae larvae were placed on A. thaliana overexpression lines (candidates Bra034774, Bra038700, Bra005919) and wild type plants and total number of hatched beetles was monitored. No differences can be seen between the age of the hatched beetles on the plants of the overexpression lines and the ones of the wild type. ●: wild type, ○: Bra034774, ▼: Bra038700, Δ: Bra005919, A and B represent two independent experiments.

Even though plant material from *A. thaliana* and gut content and RNA extracts of the gut tissue from *P. cochleariae* was collected and frozen, the analyses of these was not feasible in the limited time frame of a master thesis.

4.3 Interaction experiments

4.3.1 Test of PG activity with agarose diffusion test (ADT)

All heterologously expressed PGs were tested for their PG activity by ADT. For the ADT, equal volumes of samples were applied into small holes in agarose plates with polygalacturonic acid (PGA). When an enzyme, which is active against PGA diffuses into the plate circular around the hole, it digests the PGA around it during the incubation

time at 40 °C. Ruthenium red only binds long-chained PGA [81], so the digested spots appear unstained due to the missing binding and white halos can be seen.

The enzymes PCO_GH28-1 to 9, which were expressed by Sf9-cells, as well as the fungal enzymes AnPGII and FpPG, expressed in *P. pastoris* were tested for their activity to digest PGA (Figure 5A). As expected, PCO_GH28-1, PCO_GH28-5 and PCO_GH28-9 were active, whereas the reportedly inactive PG family members PCO_GH28-2, -3, -6 and -8 showed no activity. PCO_GH28-4 digests galacturonic acid oligomers and thus the activity cannot be detected with this plate assay [19]. Also, the fungal AnPGII and FpPG (in several dilutions) were active.

PvPGIP2, expressed in *P. pastoris*, was also applied onto the ADT and showed no PG activity (Figure 5B).

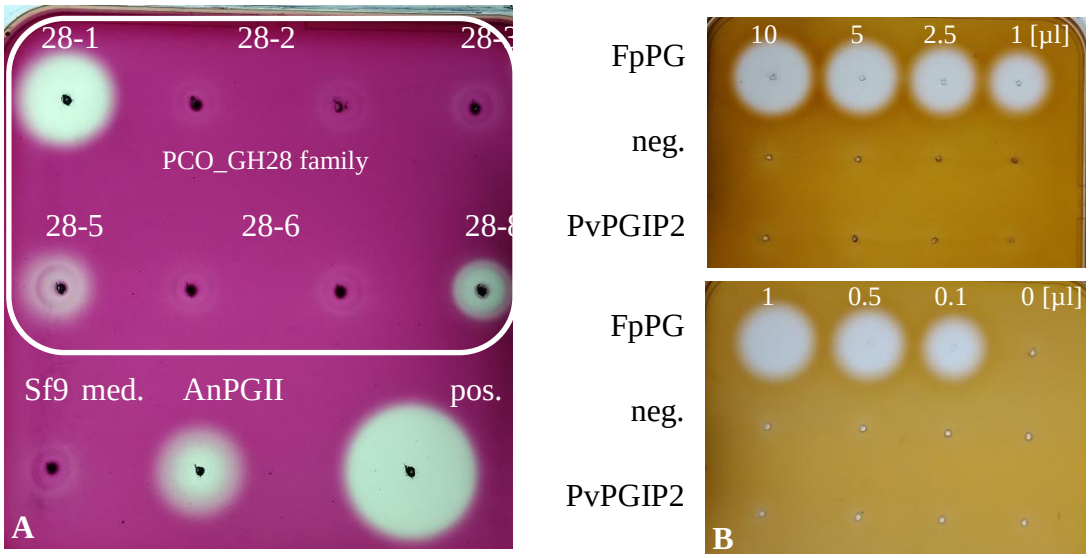

Figure 5 Agarose Diffusion Test with P. cochleariae as well as fungal PGs and PvPGIP2. A:10 µl of PCO_GH28-1, -2, -3, -4, -5, -6, -8, -9 AnPGII and FpPG were applied. A negative control: Sf9 culture medium, A positive control: PCO_GH28-1 from yeast expression, B: written volumes of FpPG, BMMY yeast medium (negative control), PvPGIP2, 10 µl total volume added up with H_2O. Visible halos for A: PCO_GH28-1, -5, -9, AnPGII and positive control; B: FpPG for 0.1-10 µl.

4.3.2 Preliminary inhibition assays with agarose diffusion tests

For inhibition assays with PGs and PGIPs, ADTs are used often [45, 57, 66]. To evaluate, if this method provides an easy and fast possibility of an inhibition assay, FpPG and PvPGIP2, were tested. This method was interesting to assess, since the work with yeast expressed proteins directly out of the BMMY medium without purification, would be helpful for unstable proteins. Especially for the putative PGIPs of *B. rapa* ssp. *pekinensis*, this method would provide great opportunities (personal communication with Wiebke Häger).

For this reason, a combination of digestion enzyme and inhibiting protein was tested by using FpPG combined with PvPGIP2 in different ratios. Both were expressed in yeast, provided by Wiebke Häger and purified by IMAC by using the His-tag, because both are very stable during purification. To exclude effects of the IMAC elution buffer on the activity of FpPG, the buffer was exchanged to citrate phosphate buffer (40 mM, pH 5.0) instead. Since PGIPs are cell-wall-associated in their natural environment, it is possible, that they protect the cell wall by laying against it. For this reason, the PGIP was applied to the plate 20 min before the PG was added, to provide the possibility to bind the PGA in the gel. Several ratios (1:0.1 – 1.8 Fp:PvPGIP) of the protein amounts between FpPG and PvPGIP2 were tested to check for an inhibition of the digestion of polygalacturonic acid by mixing a constant amount of PG with increasing amounts of PvPGIP2. (Figure 6). The inhibitor PvPGIP2 alone showed no PG activity. FpPG showed clear PG activity and was not inhibited by PvPGIP, even up to 55 ng + 99 ng PvPGIP.

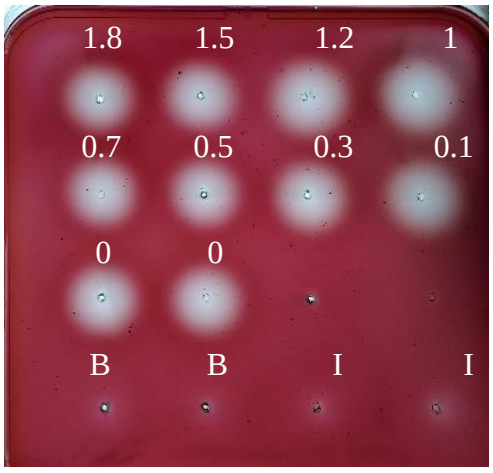

Figure 6: Agarose Diffusion Test with FpPG in combination with PvPGIP2 in different ratios. The volumes of PvPGIP2 were applied to the gel 20 min before the FpPG was added. B: 19 µl citrate phosphate buffer; I: 100 ng PvPGIP2 in citrate phosphate buffer; FpPG: 55 ng, ratios to PvPGIP2 as above; total volume always 19 µl.

Concluding, no inhibition of FpPG could be shown with ADT.

4.3.3 Establishment of interaction assay

Putative PGIPs of B. rapa ssp. pekinensis were revealed as challenging. Due to problems with aggregation of LRR-proteins and only short times of stability in experiments of the past, stable Sf9 cell lines expressing BraPGIP3_GPI (Bra005919) and PvPGIP2_GPI were established and provided by Wiebke Häger. These are membrane-anchored proteins, which are located at the outside of the cell surface

connected by a GPI anchor and in this way the contact between them is reduced and the aggregation prevented or at least minimized (personal communication with Wiebke Häger). In the natural environment, PGIPs also appear as bound molecules connected to pectin [37], not as soluble proteins, what is mimicked by this method.
This provides the possibility for an establishment of an interaction assay between PGs and putative PGIPs. This interaction assay of PGs with putative PGIPs, which offered the opportunity to work with GPI-anchored proteins expressed by Sf9 cells, was necessary to circumvent the mentioned former problems of LRR protein aggregation. For the establishment of the assay, the well-studied PG-PGIP system of FpPG and PvPGIP2 was used [67] until a successfully working method was found.

4.3.3.1 Preparation of PGs and PGIPs

FpPG was expressed in yeast and provided by Wiebke Häger. After harvesting, the enzyme was purified with IMAC, concentrated and the buffer was exchanged with H_2O. After determination of the protein concentration, a dilution series (0.1 - 5 ng) was mixed and applied to a Western Blot, whereby 5 ng of FpPG per 40 µl approach seemed to be the ideal amount for all following interaction assays, since a clear but not too strong band could have be seen.
PvPGIP2_GPI as well as wild type Sf9 plasma membrane was isolated out of Sf9 cells with differential centrifugation.

4.3.3.2 Assay conditions

Since the GPI-anchored proteins may detach from the membrane by elevated temperatures, handling on ice is necessary for all steps (Figure 7).

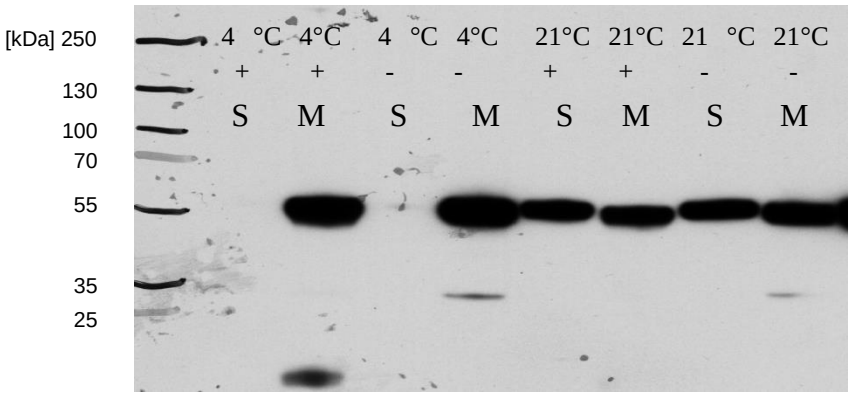

Figure 7: Western Blot test temperature impact on PvPGIP2_GPI. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI were incubated with sodium acetate buffer at 4 °C or 21 °C. One half of the samples were boiled after mixing with sample buffer (5 min, 95 °C, +). S: supernatant fraction, M: membrane fraction Detection of PGIP_myc with anti-myc antibody, exposure time: 5 min; PageRuler Plus Prestained Protein Ladder was used as size standard.

To minimize the loss of proteins, the interaction assay should be performed in protein low-binding tubes and the incubation, especially overnight, in upright position a shaking thermomixer instead of a rotating, inverting tube holder.

Since working with membrane preparations in combination with formaldehyde lead to problems like dissolving of membranes after over-night incubation, it was desirable to work with soluble proteins.

Therefor, the GPI-anchored proteins can be released from the membrane by PI-PLC treatment immediately before using them in assays. Phosphoinositide phospholipase C cleaves the GPI anchor before the phosphate group. The working concentration of 0.2 U was determined by incubating different PI-PLC concentrations (0.2 U, 2 U) at 4°C and 21°C (Supplementery Data Figure 18).

It has been shown that an incubation of PvPGIP2_GPI membrane preparation with 0.2 U PI-PLC works optimal for 1 h at room temperature (21°C).

To allow for an interaction of PG and PGIP before cross-linking and minimize unspecific interactions, a pre-incubation step of 1 h at 4°C in citrate phosphate buffer (40 mM) was added. Afterwards, an incubation at 16°C overnight with 1% formaldehyde showed the best results. Formaldehyde is used for cross-linking, because it stabilizes transient binding, to provide the possibility of its characterization.

When the proteins interact with each other, a higher shifted band (higher molecular weight) is visible. Since the proteins are tagged differently (PGIP: myc, PG: V5), the

differentiation with the Western Blot is possible. A verification with both antibodies was pursued to double check the result.

In addition, the samples shouldn't be boiled before applying them to the SDS Page, to avoid a reversing of the cross-linked bond between the PGIP_GPI and the interacting enzyme [82].

As negative controls, to avoid unspecific interactions with the Sf9 cell membrane, wild type membrane was used in the same amount (normalized with membrane protein concentration).

At first, BSA was used in the single protein controls replacing the respective interaction partner. But using it in high concentrations, additional bands in the Western Blot were observed for the anti-myc antibody. Thus, single proteins without adjusting for total proteins in the sample were used in the assays.

4.3.3.3 FpPG-PvPGIP2_GPI interaction assay

Since the interaction of FpPG with PvPGIP2 was established in 100 mM sodium acetate buffer, these conditions were used first to establish the assay. When an interaction could be shown with this method, the buffer system was switched to 40 mM citrate phosphate buffer, since that is the buffer in which all *P. cochleariae* PGs have been shown to be active.

An interaction assay between FpPG and PvPGIP_GPI using this novel technique is shown in Figure 8. PvPGIP2_GPI was detected with an anti-myc antibody (Figure 8, A) and the FpPG with an anti-V5 antibody (Figure 8, B). A band of the combined molecular weight of FpPG (approx. 65 kDa) and PvPGIP2_GPI (approx. 55 kDa) is visible at approx. 120 kDa only in the lane where both interaction partners were incubated together and cross-linked formaldehyde. No higher band was visible in the controls using the wild type membrane and the single protein controls, verifying that the interaction is specific between FpPG and PvPGIP2_GPI. Since the interaction can be seen with both antibodies targeting the PG as well as the PGIP, this further confirms that the band observed really shows the PG-PGIP cross-linked complex.

In this sample, where PvPGIP2_GPI, FpPG and formaldehyde were incubated together, it seemed as all of PvPGIP2_GPI was bound but only a part of the FpPG. In the anti-myc incubated blot, only a weak band could be seen in the lane at the molecular weight of PvPGIP2_GPI but in the anti-V5 blot, a thick band was visible, even though there

was a clear, strong band for the interaction complex, which implemented a good and successful interaction between both partners. Moreover, no unexpected signals could be seen on the blots in any of the lanes which functioned as controls, to verify, that the interaction was really between PGIP and PG. Since membrane preparations with unknown concentrations of the membrane-bound proteins were used, nothing can be assessed regarding the stoichiometry. It's only possible to verify, if an interaction exists, but nothing about the quantity.

Concluding, this newly established interaction assay can be used to qualitatively study other unknown PG-PGIP interactions.

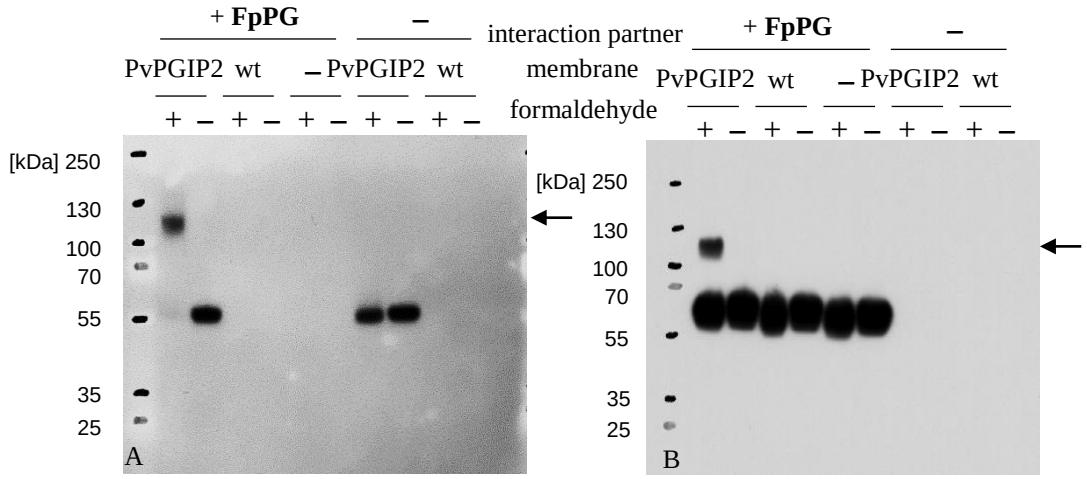

Figure 8: Western Blot of interaction assay of PvPGIP2_GPI with FpPG. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI were incubated with FpPG and cross-linked with formaldehyde. To exclude unspecific effects, FpPG was also incubated with membrane preparations from wild type cells, and all components separately as single proteins. The band of combined molecular weight of FpPG and PvPGIP is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 5 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 1 min, PageRuler Plus Prestained Protein Ladder was used as size standard.

4.3.4 Interaction assay between putative inhibitory proteins with PGs of different origins

4.3.4.1 A. niger AnPGII interaction with PvPGIP2_GPI

To further validate the applicability of the newly established assay, it was tested with another previously studied PG-PGIP combination. AnPGII has been shown to be inhibited by PvPGIP2 by D'Ovidio et al. 2004 [45]. Even though an inhibition implies an interaction, no direct interaction between the two proteins has been shown yet.

Analogous to the established PG-PGIP_GPI interaction assay, AnPGII was incubated with PvPGP2_GPI and cross-linked with formaldehyde. Instead of 5 ng FpPG, 100 ng of AnPGII was used after testing different amounts on a Western Blot to get a well visible band. The interaction is shown in Figure 9, A and B for the PGIP_myc and

PG_V5, respectively. A band of the combined molecular weight of AnPGII (approx. 58 kDa) and PvPGIP2_GPI (approx. 55 kDa) is visible at approx. 110 kDa only in the lane where both interaction partners were incubated together and cross-linked formaldehyde. Again, only one band is visible in this lane for the interaction lane at least for the anti-V5 antibody, the one on the anti-myc blot is weak, and the signal of the remaining rest of the PGIP in this lane is really intensive. This time even for the wild type membrane a little weak band is visible at the anti-V5 blot, but since the interaction band, is much more intensive and is clearly different to the control, the interaction is showed anyway cleary. According to this, the confirmation of the interaction between PvPGIP2_GPI and AnPGII could been shown as well as with FpPG and with this, the second known PG and PGIP interaction could be confirmed with this assay.

Due to problems with the antibody, the background of the anti-myc blot appeared dark, even for short times of exposure.

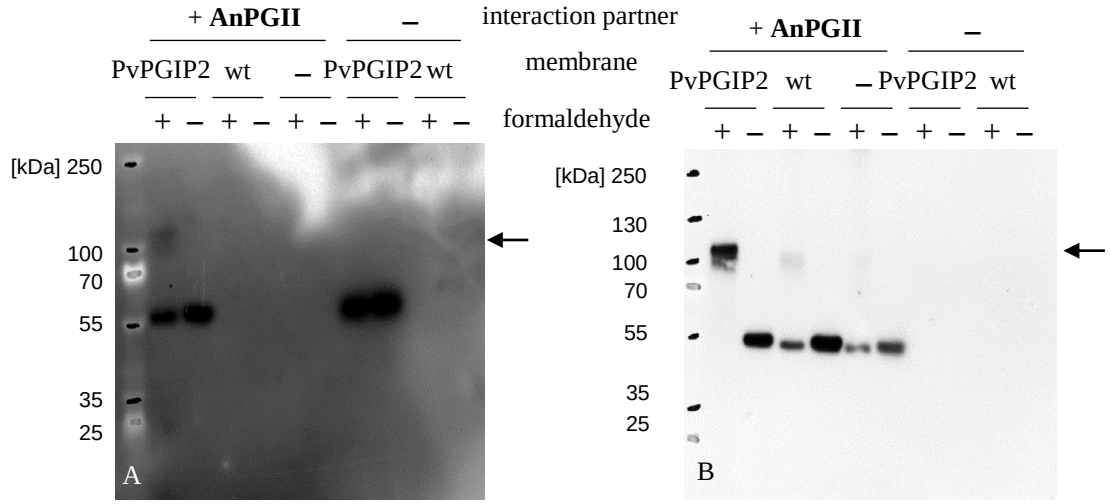

Figure 9: Western Blot of interaction assay of PvPGIP2_GPI with AnPGII. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI were incubated with AnPGII and cross-linked with formaldehyde. To exclude unspecific effects, AnPGII was also incubated with membrane preparations from wild type cells, and all components separately as single proteins. The band of combined molecular weight of AnPGII and PvPGIP is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 10 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 5 min, PageRuler Plus Prestained Protein Ladder was used as size standard.

4.3.4.2 Interaction of P. cochleariae GH28 family members with B. rapa ssp. pekinensis putative PGIP and PvPGIP2_PGI

The newly established interaction assay was used to study the interactions of all combinations of P. cochleariae GH28 family members (GH28-1, -2, -3, -4, -5, -6, -8, -9) with PvPGIP2_GPI as well as BraPGIP3 from *B. rapa* ssp. *pekinensis*.

The PGs were used directly from the Sf9 expression medium.

To avoid a possible impact of the culture medium on the interaction, an interaction assay with the positive control PvPGIP2_GPI with FpPG was carried out in this medium and showed no effect of the medium (Supplementary Data Figure 19).

As initial approach, all PG-PGIP combinations were tested just with or without formaldehyde due to the high number of samples and the feasibility of sample handling. In all blots, interacting combinations were found (Figure 10, Figure 11, Figure 12). This also shows, that the assay not only worked for PvPGIP2, but also for BraPGIP3, for which usually many problems with instability appear in soluble form. Due to the instant use after thawing of the membrane preparations, the handling time with the proteins is short and allow a structured screening of the proteins. For all samples, controls without membrane, only with enzyme were applie as well as a PvPGIP2 only sample and a BraPGIP3 only sample.

Those combinations that showed a putative positive interaction, were repeated with all proper controls. Thereof, those that were confirmed to be positive and where no bands were visible in the controls to be sure about the specifity of the interaction, are shown in the respective figures below.

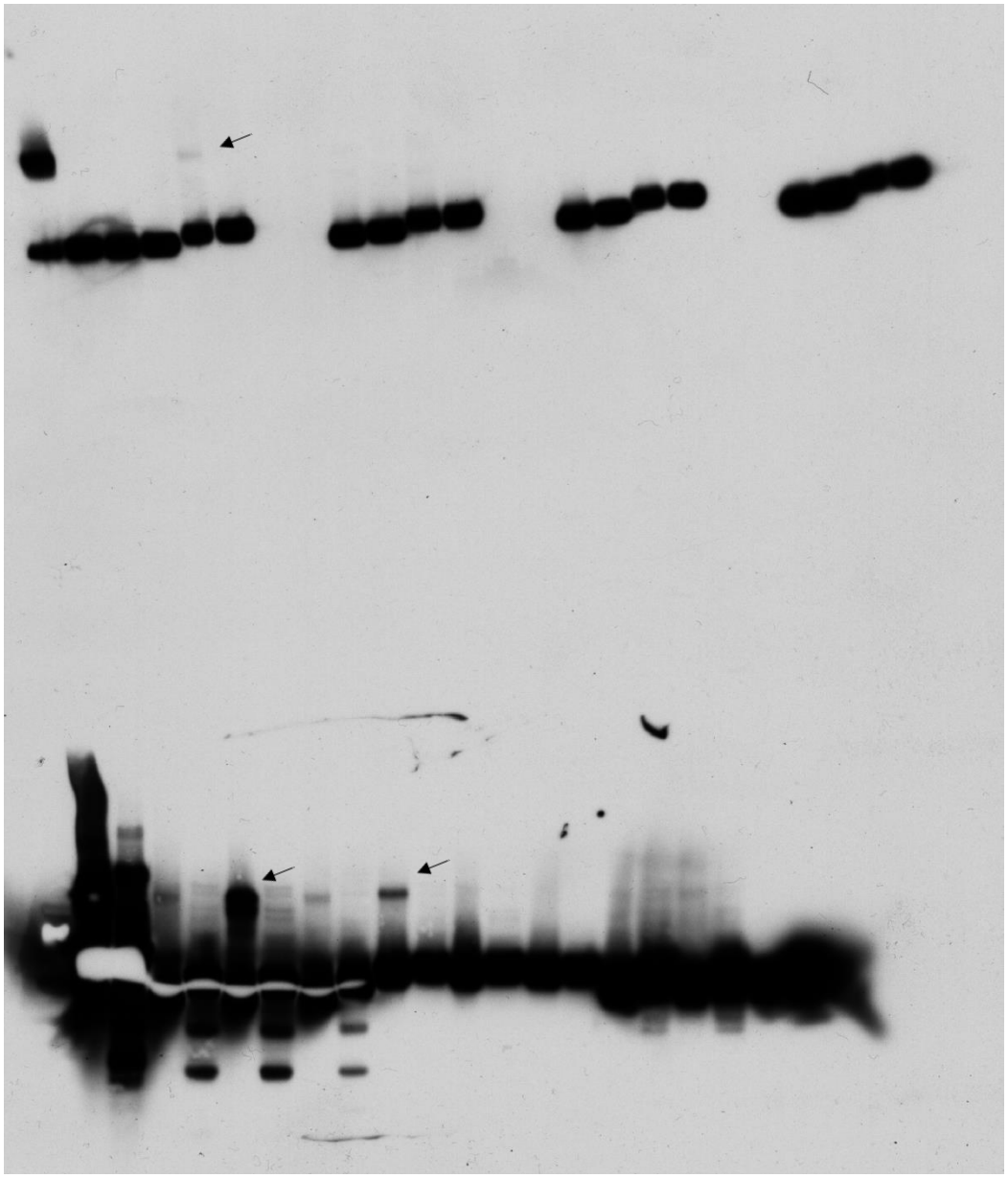

Figure 10: Western Blot of interaction assay of PvPGIP2_GPI and BraPGIP3 with PCO_GH28-1, 2, 3. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI and BraPGIP3 were incubated with PCO_GH28-1, 2, 3 and cross-linked with formaldehyde. To exclude unspecific effects, PCO_GH28-1, 2, 3. were also incubated with membrane preparations from wild type cells. The band of combined molecular weights of PvPGIP2_GPI and PCO_GH28-2 and BraPGIP3 with PCO_GH28-1 is indicated by arrows. A: detection of PGIP_myc with anti-myc antibody, exposure time: 5 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 5 min.

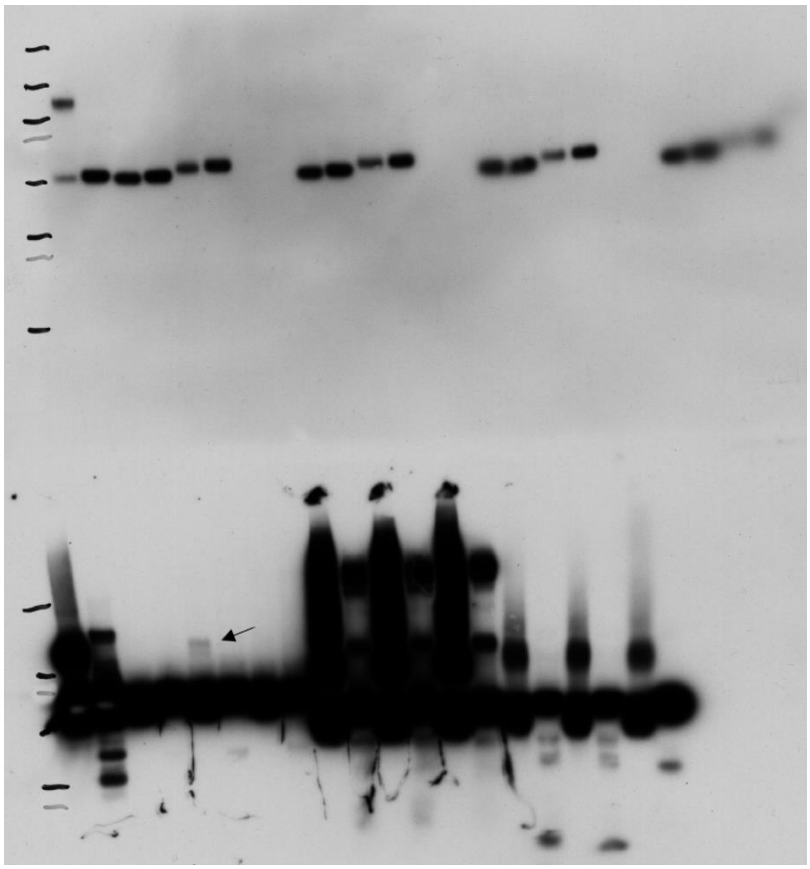

Figure 11 :Western Blot of interaction assay of PvPGIP2_GPI and BraPGIP3 with PCO_GH28-4, 5, 6. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI and BraPGIP3 were incubated with PCO_GH28-4, 5, 6 and cross-linked with formaldehyde. To exclude unspecific effects, PCO_GH28-4, 5, 6. were also incubated with membrane preparations from wild type cells. The band of combined molecular weights of PvPGIP2_GPI and PCO_GH28-4 and BraPGIP3 with PCO_GH28-1 is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 1 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 1 min. PageRuler Plus Prestained Protein Ladder was used as size standard.

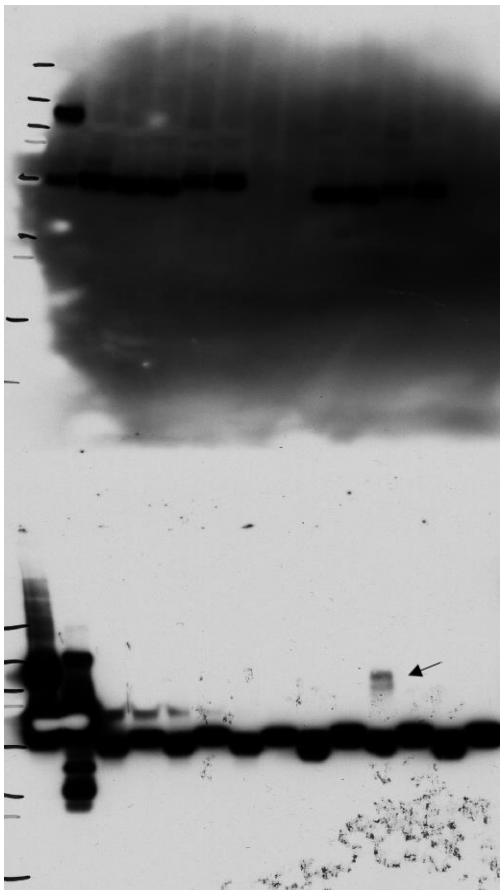

Figure 12:Western Blot of interaction assay of PvPGIP2_GPI and BraPGIP3 with PCO_GH28-8, 9. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI and BraPGIP3 were incubated with PCO_GH28-8, 9 and cross-linked with formaldehyde. To exclude unspecific effects, PCO_GH28-8, 9. were also applied without any membrane preparations. The band of combined molecular weights of BraPGIP3 with PCO_GH28-9 is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 5 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 5 min. PageRuler Plus Prestained Protein Ladder was used as size standard.

For the combination with PvPGIP2_GPI, only PCO_GH28-2 showed a band of the interaction complex. This band was not visible on the anti-myc antibody incubated blot (Figure 13, A), only on the anti-V5 antibody incubated blot (Figure 13, B) and even there, the signal was weak.

Longer exposure times were not possible due to the background signal with the anti-myc antibody and increased band area expanding in the interaction complex band space for the V5 antibody.

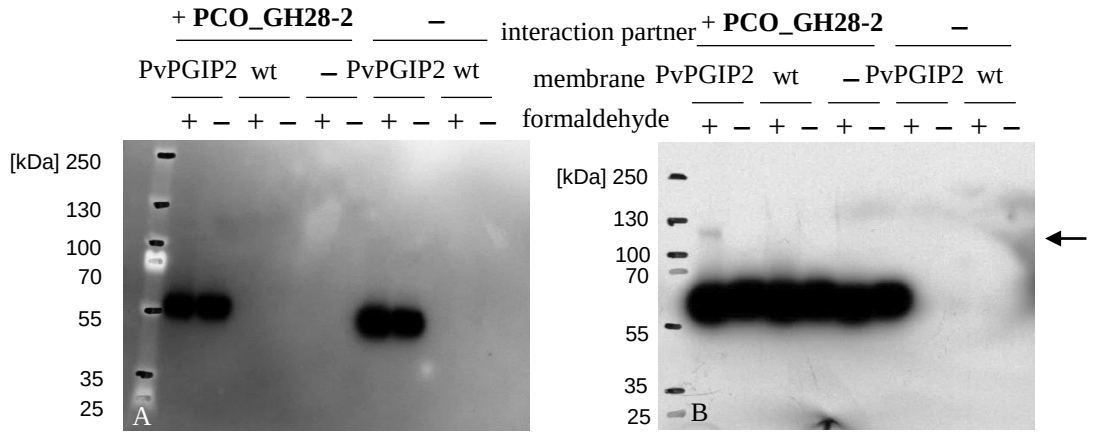

Figure 13: Western Blot of interaction assay of PvPGIP2_GPI with PCO_GH28-2. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI were incubated with PCO_GH28-2 and cross-linked with formaldehyde. To exclude unspecific effects, PCO_GH28-2 was also incubated with membrane preparations from wild type cells, and all components separately as single proteins. The band of combined molecular weight of PCO_GH28-2 and PvPGIP is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 10 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 5 min, PageRuler Plus Prestained Protein Ladder was used as size standard.

BraPGIP3_GPI from B. rapa ssp. pekinensis interacted with the P. choclearіae PGs PCO_GH28-1, -4 and -9 (Figure 19). For all three, the bands of the interaction complexes are clearly visible in the anti-V5-blot. Also for blot showing the PGIPs, small weak bands are visible for the complex, but the problem with the antibody prevented longer exposure time, so it can be only seen weakly. Again, no bands are seen in the controls, what relates to the specifity of the interaction. For the PG as well as the PGIP, there is always an additional band from remaining unbound rests visible, so the proteins are not completely bound to the interaction partner.

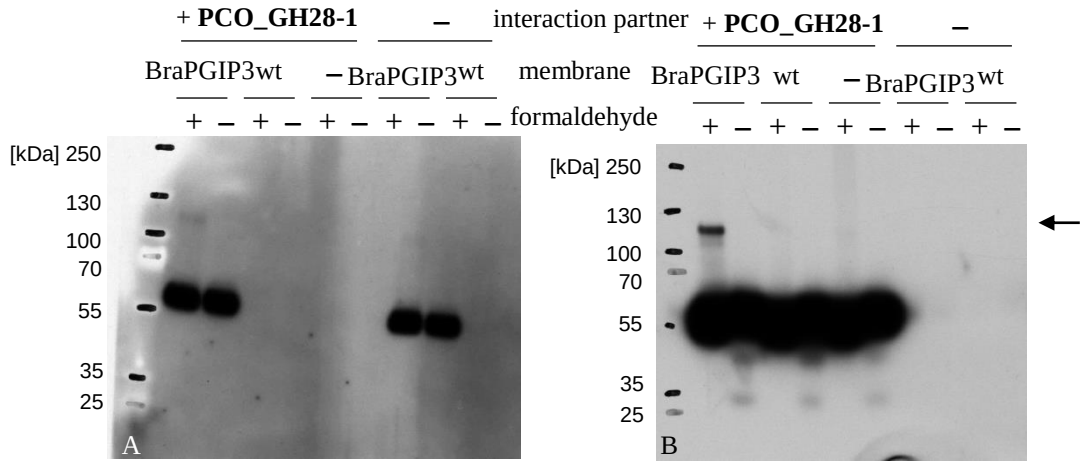

Figure 14: Western Blot of interaction assay of BraPGIP3_GPI with PCO_GH28-1. Membrane preparations from Sf9 cells expressing BraPGIP3_GPI were incubated with PCO_GH28-1 and cross-linked with formaldehyde. To exclude unspecific effects, PCO_GH28-1 was also incubated with membrane preparations from wild type cells, and all components separately as single proteins. The band of combined molecular weight of PCO_GH28-1 and BraPGIP3 is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 10 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 5 min, PageRuler Plus Prestained Protein Ladder was used as size standard.

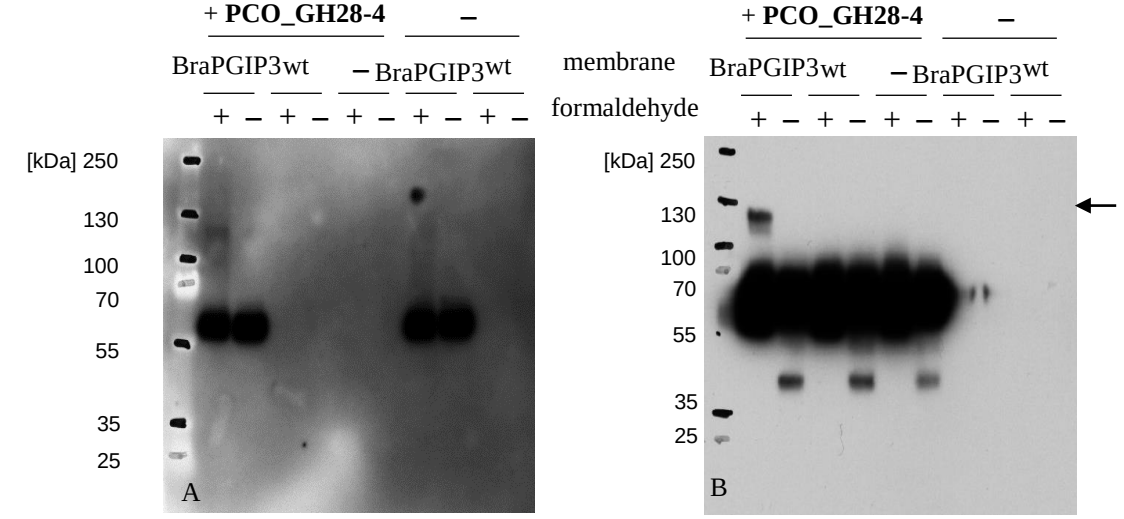

Figure 15: Western Blot of interaction assay of BraPGIP3_GPI with PCO_GH28-4. Membrane preparations from Sf9 cells expressing BraPGIP3_GPI were incubated with PCO_GH28-4 and cross-linked with formaldehyde. To exclude unspecific effects, PCO_GH28-4 was also incubated with membrane preparations from wild type cells, and all components separately as single proteins. The band of combined molecular weight of PCO_GH28-4 and BraPGIP3 is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 10 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 5 min, PageRuler Plus Prestained Protein Ladder was used as size standard.

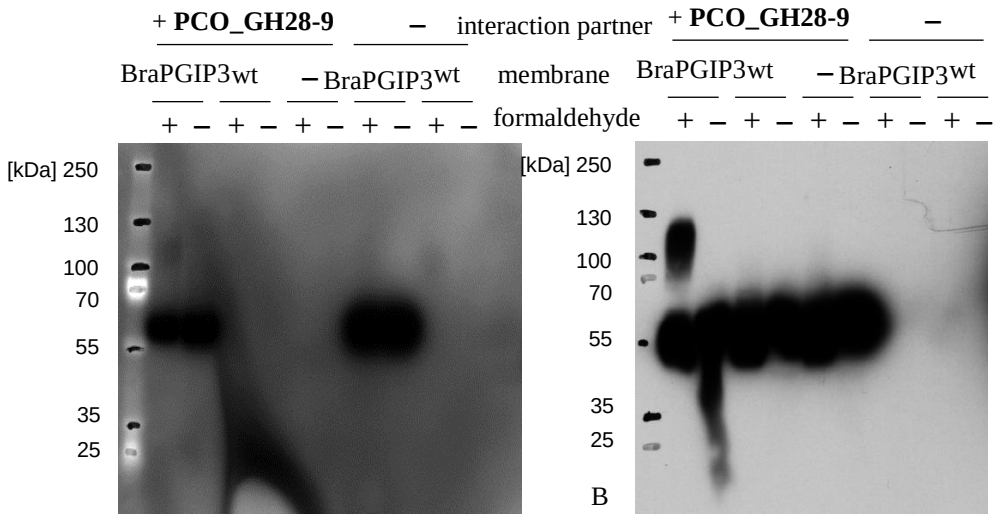

Figure 16: Western Blot of interaction assay of BraPGIP3_GPI with PCO_GH28-9. Membrane preparations from Sf9 cells expressing BraPGIP3_GPI were incubated with PCO_GH28-9 and cross-linked with formaldehyde. To exclude unspecific effects, PCO_GH28-9 was also incubated with membrane preparations from wild type cells, and all components separately as single proteins. The band of combined molecular weight of PCO_GH28-9 and BraPGIP3 is indicated by an arrow. A: detection of PGIP_myc with anti-myc antibody, exposure time: 10 min; B: detection of PG_V5 with anti-V5 antibody, exposure time: 5 min, PageRuler Plus Prestained Protein Ladder was used as size standard.

For all the other combinations of the enzymes of the GH28-familiy with both membrane proteins PvPGIP2_GPI and BraPGIP3_GPI, no interaction could be shown with the established assayin combination with both membrane proteins.

All in all, the newly established interaction assay of GPI-anchored putative PGIPs could be used to show for the first timan interaction between PGs of beetle origin with plant putative inhibitors.

5 Discussion

Plant cell walls are complex structures of cellulose, hemicellulosic and pectic polysaccharides as well as proteins [7, 8]. These polysaccharides can be targeted by PCWDEs of phytopathogens and herbivores, depolymerizing the plant cell wall [9]. Plant cell wall-associated PGIPs are known to various microbial PGs and some mirid bug PGs (Hemiptera: Miridae) [66, 83].The herbivorous beetle *P. cochleariae* possesses a variety of PCWDEs, including pectolytic enzymes of the GH28 family with three active endo-PGs (GH28-1, -5, -9), one oligogalacturonid-hydrolysing enzyme (GH28-4) and five members which show no activity against pectic substrates or other polysaccharides of the plant cell wall (GH28-2, -3, -6, -7, -8) [19]. In an interaction study of PCO_GH28-1 and PCO_GH28-3 with cell wall proteins of *B. rapa* ssp. *pekinensis*, eight putative inhibitory LRR proteins were identified [80].

Of these candidate proteins, five putative PGIPs from *B. rapa* ssp. *pekinensis* as well as PvPGIP2 from *P. vulgaris* were cloned into *A. tumefaciens*. These were used to stabily transform *A. thaliana* Col-0 wild type plants using the Floral Dip method [70, 71] . The seeds of the transformed plants were selected on agar plates with kanamycin. Successfully transformed seeds formed healthy looking plants with green leaves and stems, whereas the negative ones are smaller and of pale yellow. For each of the five candidates and PvPGIP2, more than 50 successfully transformed plants grew per two plates. This was approximately 2.5 % of all plated seeds. Compared to other transformations with *A. tumefaciens*, the transformation frequency reached here is high. Reports range between <0.2% - 3% [71, 84, 85] and are dependent on the *A. tumefaciens* strain and *A. thalina* ecotype and physiology. The surviving *A. thaliana* were genotyped by PCR from the gDNA. Apart from two plants, all tested plants (approximately 240) were positive for the respective insert after selection, which confirmed the successful transformation of nearly 100%.

Concluding, the transformation was successful for all six constructs with high transformation frequency and selection accuracy.

In *A. tumefaciens*-mediated plant transformation, the genes of interest integrate randomly in the plant genome. To verify the expression of the candidate gene-encoded proteins, Western Blots using the extracts are necessary. Due to problems with the anti-myc antibody regarding high background signals even for short exposure times, it

wasn’t possible to carry out the expression analysis of the proteins by Western Blots yet. Plant tissue and the extracted gDNA for each plant were collected and stored at -20 °C to perform this as soon as possible.
When the expression of the candidate proteins is confirmed, the creation of homozygous overexpression lines out of the created seeds could be continued.

The genes of three candidate proteins of the interaction assay [80] were previously transformed into *A. thaliana* plants and homozygous lines were created. These were used in this master thesis for a feeding assay to determine the effect of these putative PG inhibitors on growth and development of the *P. cochleariae.* Neonate larvae were placed on *A. thaliana* lines overexpressing the *B. rapa* ssp. *pekinensis* proteins Bra034774, Bra038700 and Bra005919 and wild type plants. After eleven days of feeding, all larvae were weighed.
Even though a weak trend is visible in the box plots towards slightly decreased weight for larvae feeding on Bra034774-expressing plants and slightly increased weight for Bra038700- and Bra 005919-expressing plants in comparison to the wild type plants, there was no significant difference between larvae feeding on overexpression lines and wild type.
Additionally, the development of larvae to beetles was monitored. *P. cochleariae* larvae pupate in the soil. For monitoring purposes, the plants were cut, when no larvae were present above ground. A piece of *B. rapa* ssp. *pekinensis*, the usual food in the rearing, was placed on the soil as stimulus for hatched beetles to come to the surface and refreshed regularly.
In the first attempt, the monitoring started at day 23 of the larval age, when beetles had already started hatching. That means, that the beetles of this day could have also emerged earlier. In the repetition of the feeding assay, the monitoring was started earlier, that the exact time point of the first hatching could be determined. Here, the first beetles hatched after 22 days. While the last beetles were observed after 28 days in the first approach, the period of hatching lasted until day 30 for the repetition. This was observed for all treatments and cannot be explained by feeding on a certain plant line.
Since larvae were dissected during the first approach, the total numbers of hatched beetles were higher in the second than in the first one. For the remaining larvae in both, the survival rate of the larvae was in similar range, 47-55% and 48-60%, respectively. The relatively low survival rate may be caused by the soil conditions. After the plant

was cut, the pots were not watered any more. Studies have shown that humidity of the soil can have an effect on the number of larvae that pupate and pupae survival [86]. Pupal survival was better in moist soil environments. This should be considered in potential repetitions of this experiment.
All in all, no significant differences could be observed for *P. cochleariae* in the larval weight gain nor in their development times, when feeding on wild type or overexpression lines of *A. thaliana.*

There are various possibilities why no effect of the overexpressed putative PGIPs from *B. rapa* ssp. *pekinensis* could be detected. First, the amount of expressed proteins by the transgenic plants could not have been enough to cause a phenotype. The overexpression lines have been tested positive for the expression of proteins of interest by a Western Blot. However, due to problems with the anti-myc antibody, these could not yet but should be repeated in the future. Gene expression can be quantified by qPCR using the frozen plant material collected from *A. thaliana* during the feeding experiment and compared with the natural expression levels of the two *A. thaliana* PGIPs *atpgip1* and *atpgip2*. Another explanation could be, that the putative inhibitory proteins influence *P. cochleariae*, but the beetles are able to compensate this inhibition by upregulation of the GH28 family genes or other PCWDE genes, to digest equal amounts of the transgenic plant tissue compared to the wild type plants. This could be verified by analyzing the extracted RNA of the larval gut tissue. Moreover, the collected samples of the gut content can be analyzed for potential differences in the overall gut PG activity. Also, it cannot be excluded, that the larvae fed increased amount of plant material of the overexpression lines and in this way compensated an inhibition of digestion enzymes. Samples were collected to be able to investigate all these possibilities but due to the limited time frame of the master thesis, these are still pending.
Furthermore, *P. cochleariae* possesses nine GH28 family members, of which four showed pectolytic activity. In the overexpression lines, only one putative PGIP is expressed. If this is specific for one GH28, the other ones are still active. In *B. rapa* ssp. *pekinensis*, the beetle will encounter a whole gene family of nine putative PGIPs and many PGIP-like proteins. These may work together to effectively counteract pectin digestion.
In the beetle, five of nine GH28 family members did not show any activity against pectic substrates or other plant cell wall polysaccharides, but it is possible, that they

nevertheless play a role in the digestion of plant cell walls. Kirsch et al. (2014) considered the possibility, that inactive members of the PCO_GH28 family may have the function of binding or interacting with PGIPs as “decoy” proteins, to protect the active PGs from inhibition [19].

Inhibitory activities are often tested by agarose diffusion assay [45, 87]. In this assay, PGs together with PGIPs are applied onto agarose plates with their substrate, diffuse into the agar and zones, where the enzyme has digested the substrate, can be visualized by staining. If the PG was inhibited by a PGIP, these zones are smaller than for the PG alone.

To test, if a quick testing of putative PGIPs expressed in yeast is possible with this method, the well-established combination of FpPG and PvPGIP2 was used [67]. Combining them directly from the yeast medium, did not lead to a visible inhibition. Even mimicking the natural environment with cell-wall associated PGIP attacked by external PGs by applying the PGIP to the gel some time before adding the PGs wasn’t effective. Also, combining different ratios of purified FpPG and PvPGIP with an up to 1.8-fold excess of PvPGIP did not inhibit the FpPG. It is difficult to compare the used amounts with the publications, because their activities are expressed in agarose plate units (1 unit defined as amount of protein that produces a 0.5 cm radius after 12h at 30°C). Here, the smallest possible PG amount was used that was clearly visible on the plate after 2 h at 40°C. For example, 30 ng PvPVIP are needed to inhibit 0.008 U of FpPG [60]. It is likely that the amount of PvPGIP was not enough to cause an inhibition of FpPG here. Also, the tests were carried out in citrate phosphate buffer and not in sodium acetate buffer like previously reported. Probably the conditions in the agar diffusion test weren’t chosen optimally for the tested proteins. Many more tests and fine tuning of the agarose diffusion assay would have to be done to reproduce this method properly to achieve better results. To quickly test PGIP activity directly from the culture medium, this method is unsuitable.

A more precise method compared to inhibition zone measuring on an agarose plate is quantifying the release of reducing sugars from the substrate. This is not possible directly from the culture medium because the sugar in the medium causes too high background signals.

Unfortunately, the putative PGIPs from *B. rapa* ssp. *pekinensis* are lost during dialysis or buffer exchange on a column, probably due to aggregation (personal communication

with Wiebke Häger) and cannot be used like this to quantify the inhibition of PGs by PGIPs.

The aggregation of LRR proteins can be minimized by expressing them as membrane-anchored proteins on the outside of the cell surface, which reduces the contact between them. In the natural environment, PGIPs also appear as bound molecules connected to pectin [37], not as soluble proteins, what is mimicked by this method.

An interaction assay of PGs with PGIPs was established with PvPGIP2_GPI and FpPG and then applied to new interactions of PvPGIP2_GPI and BraPGIP3_GPI (Bra005919) with PGs of various origins.

The GPI-anchored proteins were released shortly before the interaction assay with the PGs from the membrane by GPI anchor cleavage. They were then pre-incubated for 1 h with the PGs and then cross-linked with formaldehyde overnight.

Both PvPGIP2 and BraPGIP3 were stable for the duration of the interaction assay (approximately 20 h) and could be detected in the Western Blot successfully afterwards. The soluble BraPGIP3 from the yeast medium aggregated and was not detectable after one day (personal communication with Wiebke Häger).

A band of higher molecular weight was detected, when the PvPGIP2 and FpPG were cross-linked with formaldehyde, but not in the controls. Thus, the interaction assay was successfully established. This also confirmed the interaction, shown by Benedetti et al. [67]. This established assay lays the basis for the testing of many different PG-PGIP combinations.

AnPGII of *A. niger* has been shown to be inhibited by PvPGIP2 [45]. This interaction could be reproduced and confirmed with this new interaction assay. Both interactions could be proven by visible shifted bands in the Western Blots with two different antibodies against both, the PG and the PGIP, confirming the specificity of the interaction.

However, since no purified PGIPs but membrane preparations were used, you cannot determine the stoichiometry of the interaction partners and only see if proteins are interacting or not.

Afterwards, the assay was used to qualitatively evaluate unknown combinations of different (putative) PGIPs with PGs or PCO_GH28 family members regarding possible interactions. Therefor, PvPGIP2_GPI as well as BraPGIP3_GPI were tested in all combinations with FpPG, AnPGIIand PCO_GH28-1, -2, -3, -4, -5, -6, -8, -9. For the combinations of PvPGIP2_GPI with PCO_GH28-2 as well as for BraPGIP3_GPI with

PCO_GH28-1, -4 and -9 interactions between the proteins could be seen, for all at least in the anti-V5-incubated blots, in some cases also in the anti-myc incubated blots. For none of the Western Blot controls, any additional bands were visible, indicating that the shown interaction are specifically between the used proteins.

In conclusion, a new interaction assay was established to work with soluble unstable proteins by using their expression as GPI-anchored membrane proteins as basis, which offers the possibility of structured screening in combination with potential interaction partners. Not only this method was used for the confirmation of already known interactions, but it was also used to successfully show novel interactions of PvPGIP2_GPI and BraPGIP3_GPI with several members of the GH28-family of *P. cochleariae* It is the first time an interaction of putative plant PGIPs and PGs of beetle origin was shown.
The stable nature of the GPI-anchored proteins also makes it possible to test in the future, if BraPGIP3_GPI is really inhibiting the PGs that they interact with.

Since interactions of BraPGIP3_GPI with all active PGs except PCO_GH28-5, but not with any inactive GH28-family member, were shown in the assay, the hypothesis of the inactive GH28 family members functioning as "decoy" for the PGIPs [19] could not be confirmed for the tested proteins. The role of the inactive GH28 family members still remains unknown. However, it still has to be confirmed by inhibition assays, that BraPGIP3 is really a PG-inhibiting protein.

Interestingly, GH28-2, one of the inactive GH28-2 family members, interacted with PvPGIP2_GPI. A possible explanation can be, that this enzyme is not an original beetle enzyme but of fungal origin, gained by horizontal gene transfer in the past but without an active role in the bettle anymore.

PCO_GH28-5, in contrast to all other active PGs from *P. cochleariae*, could not be shown to interact with BraPGIP3_GPI. Only one of the various *B. rapa* ssp. *pekinensis* putative PGIPs was tested. Since PGs and PGIPs are hypothesized to have been formed by an evolutionary arms race [41], it may be possible that it interacts with any of the other LRR proteins.

Considering the results of the interaction assay, the interpretation of the feeding assay can be reassessed. As shown, at three out of four active GH28s of *P. cochleariae* interact with BraPGIP3_GPI, which was expressed in the Bra005919 *A. thaliana* overexpression line. Feeding on these plants caused no difference in weight gain and development time of the larvae compared to the wild type. The results from the interaction assay favour the explanations of upregulated PG expression in the beetle or too low Bra005919 expression levels in the plants. It is less likely, that inactive GH28 family members work as "decoy" for the putative PGIP. Even though Bra005919 interacts with the active PGs, it still has to be shown that it is really inhibiting them, since this could also be a reason that no effect on the beetle could be shown.

The other putative PGIPs from *B. rapa* ssp. *pekinensis* could not yet be expressed as GPI-anchored proteins in Sf9 cells. It would be interesting to test all candidate proteins with the *P. cochleariae* PGs. Since an interaction could be shown for Bra005919 with PCO_GH28-1, -4 and -9, these can be used to establish another kind of interaction assay to test those proteins for which the GPI-anchored expression does not work.

PGIPs are ionically bound to the cell wall [37]. The whole cell walls, including the bound proteins could be extracted from the plants to enrich the expressed proteins and keep them bound to the cell wall to maybe stabilize them. The whole cell wall with the bound proteins can be tested in comparison to the wild type cell walls, if they bind to GH28 family members, analogous to the GPI-anchored interaction assay. This could help to investigate all putative PGIPs from *B. rapa* ssp. *pekinensis*.

All in all, the establishment of the interaction assay based on membrane-anchored candidate proteins offers the possibility to adapt this method in many different ways to study protein-protein interactions and inhibition assays including instable partners, that are otherwise challenging to work with.

6 Future perspectives

In this master thesis, five putative PGIPs of *B. rapa* ssp. *pekinensis* were transformed in *A. thaliana* plants. Moreover, a feeding assay was carried out with *P. cochleariae* feeding on three *A. thaliana* overexpression lines of other putative PGIPs of *B. rapa* ssp. *pekinensis*. No significant differences could be recognized, neither in growth (weight gain) nor in the development from larvae to beetle. This feeding assay could be repeated with the other five candidates, when homozygous plants of them are reared.

For a potential explanation of no significant differences, the collected samples of the plants and the gut tissue could be used for gene expression analyses. In addition, with the gut content, PG activity assays can be carried out to analyse, if the protein expression in the plants was too low or if *P. cochleariae* has possibilities to compensate an inhibiting effect of the putative PGIPs. Furthermore, it has to be shown, if the expressed candidates are the related inhibitors to the beetle PGs.

Besides, a new interaction assay was established. Already known interactions (PvPGIP2 with FpPG and AnPGII) have been confirmed and new unknown interactions have been shown. As a result, an interaction between beetle PGs and putative PGIP of plants was demonstrated successfully the first time. Moreover, the assay offers the opportunity to work with instable proteins and in this way, provides the opportunity for many further tests for interactions. Potentially this interaction assay could be developed into an inhibition assay. Maybe it will become also possible, to screen the other candidates with the established interaction assay by using isolated cell walls analogously to the GPI-anchored proteins of the Sf9 cell plasma membrane.

The establishment of the interaction assay is the basis for many further tests, that will provide the opportunity to expand the knowledge about the gene families of PGIPs and PGs.

7 Summary

Plant cell walls are structural and functional complex, but they are generally composed of about 10 % of proteins and approximately 90 % of polysaccharides, such as cellulose, hemicellulose as well as pectic polysaccharides. [1, 2, 6-8]. These cell wall polysaccharides are the target for many plant cell wall degrading enzymes (PCWDE), secreted by phytopathogenic microorganisms like fungi, bacteria, and nematodes. Out of these, pectinases, especially poylgalacturonases (PGs), are the first enzymes produced during a plant infection and are generally regarded as important pathogenicity factors for many plant pathogens [14-16]. The mustard leaf beetle *Phaedon cochleariae* is an insect pest species of the Chrysomelidae family [33] [34]. Of the GH 28 family, *P. cochleariae* possesses nine enzymes. Three of them are active as endo-PGs (GH28-1, -5, -9), GH28-4 is active as an oligogalacturonase, the remaining five (GH28-2, -3, -6, -7, -8) show no activity against any of the tested pectic substrates or other polysaccharides of the plant cell wall [19, 36]. One way to cope with PCWDEs is the production of proteininacious inhibitors, e.g. polygalacturonase-inhibiting proteins (PGIPs). These are extracellular plant proteins, which are bound to homogalacturonan [37] and inhibit PGs in their acitivity to digest pectin. In a previous interaction study, eight proteins of *B. rapa* ssp. *pekinensis* were identified as interaction partners and possible inhibitors of the beetle enzymes PCO_GH28-1 and PCO_GH28-3 (master thesis Wiebke Häger). For five of these eight candidates (Bra035741, Bra005917, Bra005917+9nt, Bra009238, Bra005916) and PvPGIP2 a transformation in the model plant *A. thaliana* was successfully performed in this master thesis. For the other three candidates, a feeding assay with P. cochleariae was carried out. The weight gain and development of its larvae was monitored for the feeding on three overexpression lines in comparison to larvae feeding on wild type *A. thaliana* plants. No significant differences could be shown, but gut tissue, gut content and plant tissue was collected fur further analyses of potential explanations.

Moreover, an interaction assay was established to circumvent former aggregation problems with putative PGIPs of *B. rapa* ssp. *pekinensis.* The assay was successfully established, two already known interactions could be confirmed and several further, unknown interactions were demonstrated. Particularly mentioned should be the first shown interaction of beetle PGs with a plant PGIP. With this assay it was possible to screen even unstable PGIPs structured and without problems.

8 Zusammenfassung

Pflanzenzellwände sind ein struktureller und funktioneller Komplex, bestehen aber in der Regel aus etwa 10 % Proteinen und etwa 90 % Polysacchariden wie Cellulose, Hemicellulose sowie pektischen Polysacchariden. [1, 2, 6-8]. Diese Zellwandpolysaccharide sind das Ziel vieler Pflanzenzellwand abbauender Enzyme (PCWDE), die von phytopathogenen Mikroorganismen wie Pilzen, Bakterien und Nematoden abgesondert werden. Pektinasen, insbesondere Poylgalacturonasen (PGs), sind die ersten Enzyme, die während einer Pflanzeninfektion produziert werden und gelten allgemein als wichtige Pathogenitätsfaktoren für viele Pflanzenpathogene[14-16]. Der Senfblattkäfer Phaedon cochleariae ist eine Insektenschädlingsart aus der Familie der Chrysomelidae[33][34]. Aus der Familie der GH 28 besitzt P. cochleariae neun Enzyme. Drei von ihnen sind als Endo-PGs aktiv (GH28-1, -5, -9), GH28-4 ist als Oligogalacturonase aktiv, die restlichen fünf (GH28-2, -3, -6, -7, -8) zeigen keine Aktivität gegen eines der getesteten pektischen Substrate oder andere Polysaccharide der Pflanzenzellwand [19, 36]. Eine Möglichkeit, mit PCWDEs umzugehen, ist die Produktion proteinhaltiger Inhibitoren, z.B. Polygalacturonase-inhibierender Proteine (PGIPs). Dabei handelt es sich um extrazelluläre Pflanzenproteine, die an Homogalacturonan[37] gebunden sind und PGs in ihrer Aktivität zum Verdauen von Pektin hemmen. In einer früheren Interaktionsstudie wurden acht Proteine von B. rapa ssp. pekinensis als Interaktionspartner und mögliche Inhibitoren der Käferenzyme PCO_GH28-1 und PCO_GH28-3 identifiziert (Masterarbeit Wiebke Häger). Für fünf dieser acht Kandidaten (Bra035741, Bra005917, Bra005917+9nt, Bra009238, Bra005916) und PvPGIP2 wurde in dieser Masterarbeit eine Transformation in die Modellpflanze A. thaliana erfolgreich durchgeführt. Für die anderen drei Kandidaten wurde ein Feeding Assay mit P. cochleariae durchgeführt. Die Gewichtszunahme und Entwicklung der Larven auf den drei Überexpressionslinien im Vergleich zu den Larven auf Wildtyp A. thaliana Pflanzen beobachtet. Es konnten keine signifikanten Unterschiede festgestellt werden, aber Darmgewebe, Darminhalt und Pflanzengewebe wurden für weitere Analysen möglicher Erklärungen gesammelt.

Darüber hinaus wurde ein Interaktionsassay etabliert, um frühere Aggregationsprobleme mit vermeintlichen PGIPs von B. rapa ssp. pekinensis zu umgehen. Der Assay wurde erfolgreich etabliert, zwei bereits bekannte Interaktionen konnten bestätigt werden und

weitere, unbekannte Interaktionen wurden nachgewiesen. Besonders hervorzuheben ist die erste gezeigte Interaktion von Käfer-PGs mit einem pflanzlichen PGIP. Mit diesem Assay war es möglich, auch instabile PGIPs strukturiert und problemlos zu überprüfen.

9 Literature

1. Caffall, K.H. and D. Mohnen, *The structure, function, and biosynthesis of plant cell wall pectic polysaccharides.* Carbohydr Res, 2009. **344**(14): p. 1879-900.
2. Brett, C.W., K. W., *Physiology and Biochemistry of Plant Cell Walls*. Vol. 2. 1990: Springer Netherlands.
3. Popper, Z.A. and S.C. Fry, *Primary cell wall composition of bryophytes and charophytes.* Ann Bot, 2003. **91**(1): p. 1-12.
4. Showalter, A.M., *Structure and function of plant cell wall proteins.* Plant Cell, 1993. **5**(1): p. 9-23.
5. Keegstra, K., *Plant cell walls.* Plant Physiol, 2010. **154**(2): p. 483-6.
6. Harholt, J., A. Suttangkakul, and H. Vibe Scheller, *Biosynthesis of pectin.* Plant Physiol, 2010. **153**(2): p. 384-95.
7. Juge, N., *Plant protein inhibitors of cell wall degrading enzymes.* Trends Plant Sci, 2006. **11**(7): p. 359-67.
8. McNeil, M., et al., *Structure and function of the primary cell walls of plants.* Annu Rev Biochem, 1984. **53**: p. 625-63.
9. Walton, J.D., *Deconstructing the Cell Wall.* Plant Physiol, 1994. **104**(4): p. 1113-1118.
10. ten Have, A., et al., *The endopolygalacturonase gene Bcpg1 is required for full virulence of Botrytis cinerea.* Mol Plant Microbe Interact, 1998. **11**(10): p. 1009-16.
11. Dong, Z. and Z. Wang, *Isolation and heterologous expression of a polygalacturonase produced by Fusarium oxysporum f. sp. cubense race 1 and 4.* Int J Mol Sci, 2015. **16**(4): p. 7595-607.
12. Huang, Q.A., C., *Polygalacturonases are required for rapid colonization and full virulence of Ralstonia solanacearum on tomato plants.* Physiological and Molecular Plant Pathology, 2000. **57**(2): p. 77-83.
13. Kubicek, C.P., T.L. Starr, and N.L. Glass, *Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi.* Annu Rev Phytopathol, 2014. **52**: p. 427-51.
14. Alghisi, P.F., F., *Pectin-degrading enzymes and plant-parasite interactions.* European Journal of Plant Pathology, 1995. **101**: p. 365-375.
15. Annis, S.L.G., P. H., *Recent advances in the molecular genetics of plant cell wall-degradingenzymes produced by plant pathogenic fungi.* European Journal of Plant Pathology, 1997. **103**: p. 1-14.
16. Kalunke, R.M., et al., *An update on polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein that protects crop plants against pathogens.* Front Plant Sci, 2015. **6**: p. 146.
17. Calderón-Cortés, N.Q., M.; Watanabe, H.; Cano-Camacho, H.; Oyama, K., *Endogenous Plant Cell Wall Digestion: A Key Mechanism in Insect Evolution.* Annual Review of Ecology, Evolution, and Systematics 2012. **43**: p. 45-71.
18. Martin, M.M., *The evolution of cellulose digestion in insects.* Philosophical Transactions: Biological Sciences, 1991. **333**: p. 281-288.
19. Kirsch, R., et al., *Horizontal gene transfer and functional diversification of plant cell wall degrading polygalacturonases: Key events in the evolution of herbivory in beetles.* Insect Biochem Mol Biol, 2014. **52**: p. 33-50.
20. Watanabe, H., et al., *A cellulase gene of termite origin.* Nature, 1998. **394**(6691): p. 330-1.

21. Shelomi, M., H. Watanabe, and G. Arakawa, *Endogenous cellulase enzymes in the stick insect (Phasmatodea) gut.* J Insect Physiol, 2014. **60**: p. 25-30.
22. Pauchet, Y., et al., *Diversity of beetle genes encoding novel plant cell wall degrading enzymes.* PLoS One, 2010. **5**(12): p. e15635.
23. Kirsch, R., et al., *Combining proteomics and transcriptome sequencing to identify active plant-cell-wall-degrading enzymes in a leaf beetle.* BMC Genomics, 2012. **13**: p. 587.
24. Jaubert, S., et al., *A polygalacturonase of animal origin isolated from the root-knot nematode Meloidogyne incognita.* FEBS Lett, 2002. **522**(1-3): p. 109-12.
25. McKenna, D.D., et al., *The evolution and genomic basis of beetle diversity.* Proc Natl Acad Sci U S A, 2019.
26. Pauchet, Y. and D.G. Heckel, *The genome of the mustard leaf beetle encodes two active xylanases originally acquired from bacteria through horizontal gene transfer.* Proc Biol Sci, 2013. **280**(1763): p. 20131021.
27. Danchin, E.G., et al., *Multiple lateral gene transfers and duplications have promoted plant parasitism ability in nematodes.* Proc Natl Acad Sci U S A, 2010. **107**(41): p. 17651-6.
28. Strong, D.R.L., J. H.; Southwood, T. R. E., *Insects on Plants*. 1984: Harvard Univ. Press.
29. Ślipiński, S.A.L., R. A. B.; Lawrence, J. F, in *Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness*, Z.-Q. ZHANG, Editor. 2011. p. 203-208.
30. PM, H., *Species Inventory. Global Biodiversity. Status of the Earth'sLiving Resources. A Report Compiled by the World Conservation Monitoring Centre,ed Groombridge B.* 1992, London: Chapman and Hall, London.
31. Dettner, K.P., W., *Lehrbuch der Entomologie*. 2011: Spektrum Akademischer Verlag.
32. Farrell, B.D., *"Inordinate Fondness" explained: why are there So many beetles?* Science, 1998. **281**(5376): p. 555-9.
33. Finch, S.J., T. H., *Interspecific competition during host plant selection by insect pests of cruciferous crops.* Insect-Plants, 1987: p. 85-90.
34. Uddin, M.M., C. Ulrichs, and I. Mewis, *Phaedon cochleariae (F.) performance on different crucifer varieties with different glucosinolate profiles.* Commun Agric Appl Biol Sci, 2008. **73**(3): p. 563-72.
35. Girard, C. and L. Jouanin, *Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle, Phaedon cochleariae.* Insect Biochem Mol Biol, 1999. **29**(12): p. 1129-42.
36. Kirsch, R., et al., *Pectin Digestion in Herbivorous Beetles: Impact of Pseudoenzymes Exceeds That of Their Active Counterparts.* Front Physiol, 2019. **10**: p. 685.
37. Spadoni, S., et al., *Polygalacturonase-inhibiting protein interacts with pectin through a binding site formed by four clustered residues of arginine and lysine.* Plant Physiol, 2006. **141**(2): p. 557-64.
38. Toubart, P., et al., *Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of Phaseolus vulgaris L.* Plant J, 1992. **2**(3): p. 367-73.
39. *https://www.ncbi.nlm.nih.gov/*. cited at: 30.11.19].
40. *https://www.uniprot.org/*. cited at: 30.11.19].
41. Casasoli, M., et al., *Integration of evolutionary and desolvation energy analysis identifies functional sites in a plant immunity protein.* Proc Natl Acad Sci U S A, 2009. **106**(18): p. 7666-71.

42. De Lorenzo, G., R. D'Ovidio, and F. Cervone, *The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi.* Annu Rev Phytopathol, 2001. **39**: p. 313-35.
43. Hegedus, D.D., et al., *Brassica napus possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to Sclerotinia sclerotiorum infection, wounding and defense hormone treatment.* Planta, 2008. **228**(2): p. 241-53.
44. Ferrari, S., et al., *Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection.* Plant Cell, 2003. **15**(1): p. 93-106.
45. D'Ovidio, R., et al., *Characterization of the complex locus of bean encoding polygalacturonase-inhibiting proteins reveals subfunctionalization for defense against fungi and insects.* Plant Physiol, 2004. **135**(4): p. 2424-35.
46. Li, R., et al., *Two Brassica napus polygalacturonase inhibitory protein genes are expressed at different levels in response to biotic and abiotic stresses.* Planta, 2003. **217**(2): p. 299-308.
47. Lagaert, S., T. Belien, and G. Volckaert, *Plant cell walls: Protecting the barrier from degradation by microbial enzymes.* Semin Cell Dev Biol, 2009. **20**(9): p. 1064-73.
48. Devoto, A., et al., *The promoter of a gene encoding a polygalacturonase-inhibiting protein of Phaseolus vulgaris L. is activated by wounding but not by elicitors or pathogen infection.* Planta, 1998. **205**(2): p. 165-74.
49. Hwang, B.H., Bae, H., Lim, HS. et al., *Overexpression of polygalacturonase-inhibiting protein 2 (PGIP2) of Chinese cabbage (Brassica rapa ssp. pekinensis) increased resistance to the bacterial pathogen Pectobacterium carotovorum ssp. carotovorum.* Plant Cell, Tissue and Organ Culture (PCTOC), 2010. **103**(3): p. 293-305.
50. Doostdar, H.M., T.G.; Mayer, R.T., *Purification and Characterization of an endo-Polygalacturonase from the Gut of West Indies Sugarcane Rootstalk Borer Weevil (Diaprepes abbreviatus L.) Larvae.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 1997. **118**(4): p. 861-867.
51. Cervone, F.D.L., G.; Pressey, R.; Darvill, A.G.; Albersheim, P., *Can Phaseolus PGIP inhibit pectic enzymes from microbes and plants?* Phytochemistry, 1990. **29**(2): p. 447-449.
52. Darvill, A., et al., *Oligosaccharins--oligosaccharides that regulate growth, development and defence responses in plants.* Glycobiology, 1992. **2**(3): p. 181-98.
53. Farmer, E.E., et al., *Oligosaccharide signaling in plants. Specificity of oligouronide-enhanced plasma membrane protein phosphorylation.* J Biol Chem, 1991. **266**(5): p. 3140-5.
54. Garcia-Brugger, A., et al., *Early signaling events induced by elicitors of plant defenses.* Mol Plant Microbe Interact, 2006. **19**(7): p. 711-24.
55. Powell, A.L., et al., *Transgenic expression of pear PGIP in tomato limits fungal colonization.* Mol Plant Microbe Interact, 2000. **13**(9): p. 942-50.
56. Ferrari, S., et al., *Transgenic expression of a fungal endo-polygalacturonase increases plant resistance to pathogens and reduces auxin sensitivity.* Plant Physiol, 2008. **146**(2): p. 669-81.

57. Janni, M., et al., *The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen Bipolaris sorokiniana.* Mol Plant Microbe Interact, 2008. **21**(2): p. 171-7.
58. Borras-Hidalgo, O., et al., *A gene for plant protection: expression of a bean polygalacturonase inhibitor in tobacco confers a strong resistance against Rhizoctonia solani and two oomycetes.* Front Plant Sci, 2012. **3**: p. 268.
59. Desiderio, A., et al., *Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in Phaseolus vulgaris.* Mol Plant Microbe Interact, 1997. **10**(7): p. 852-60.
60. Leckie, F., et al., *The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed beta-strand/beta-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability.* EMBO J, 1999. **18**(9): p. 2352-63.
61. Jones, J.D., *Putting knowledge of plant disease resistance genes to work.* Curr Opin Plant Biol, 2001. **4**(4): p. 281-7.
62. Stotz, H.U., et al., *Structure and expression of an inhibitor of fungal polygalacturonases from tomato.* Plant Mol Biol, 1994. **25**(4): p. 607-17.
63. Kobe, B. and A.V. Kajava, *The leucine-rich repeat as a protein recognition motif.* Curr Opin Struct Biol, 2001. **11**(6): p. 725-32.
64. King, D., et al., *Use of amide exchange mass spectrometry to study conformational changes within the endopolygalacturonase II-homogalacturonan-polygalacturonase inhibiting protein system.* Biochemistry, 2002. **41**(32): p. 10225-33.
65. Federici, L., et al., *Structural requirements of endopolygalacturonase for the interaction with PGIP (polygalacturonase-inhibiting protein).* Proc Natl Acad Sci U S A, 2001. **98**(23): p. 13425-30.
66. Frati, F.G., R.; De Lorenzo, G.; Salerno, G.; Conti, E, *Activity of endo-polygalacturonases in mirid bugs (Heteroptera: Miridae)and their inhibition by plant cell wall proteins (PGIPs).* European Journal of Entomology, 2006. **103**: p. 515-522.
67. Benedetti, M., et al., *Structural resolution of the complex between a fungal polygalacturonase and a plant polygalacturonase-inhibiting protein by small-angle X-ray scattering.* Plant Physiol, 2011. **157**(2): p. 599-607.
68. Joubert, D.A., et al., *A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from Botrytis cinerea in Nicotiana benthamiana leaves without any evidence for in vitro interaction.* Mol Plant Microbe Interact, 2007. **20**(4): p. 392-402.
69. Zhu, G., et al., *ZmPGIP3 Gene Encodes a Polygalacturonase-Inhibiting Protein that Enhances Resistance to Sheath Blight in Rice.* Phytopathology, 2019. **109**(10): p. 1732-1740.
70. Bernhardt, K.V., S.K. ; Wiese, J.; Linka, N.; Weber, A.P.M., *Agrobacterium-mediated Arabidopsis thaliana transformation: an overview of T-DNA binary vectors, floral dip and screening for homozygous lines.* Journal of Endocytobiosis and Cell Research, 2012: p. 19-28.
71. Harrison, S.J., et al., *A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation.* Plant Methods, 2006. **2**: p. 19.
72. Nour-Eldin, H.H., et al., *Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments.* Nucleic Acids Res, 2006. **34**(18): p. e122.

73. Research, Z. *https://files.zymoresearch.com/protocols/_d4003t_d4003_d4004_d4013_d4014_dna_clean_concentrator_-5.pdf*. cited at: 30.11.19].
74. Scientific, T. *https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013117_GeneJET_Plasmid_Miniprep_UG.pdf*. cited at: 30.11.19].
75. Scientific, T. *https://www.thermofisher.com/de/de/home/references/protocols/nucleic-acid-purification-and-analysis/dna-extraction-protocols/purelink-hipure-plasmid-filter-purification-kits.html#prot4*. cited at: 30.11.19].
76. Jena, A. *https://www.analytik-jena.de/fileadmin/content/products/02_Kits/innuPREP_RNA_Mini_Kit_2_0/Manual_innuPREP_RNA_Mini_Kit_2_0_en.pdf*. cited at: 30.11.19].
77. Research, Z. *https://files.zymoresearch.com/protocols/_r1013_r1014_r1015_r1016_rna_clean_concentrator-5.pdf*. cited at: 30.11.19].
78. Merckmillipore. *http://www.merckmillipore.com/DE/de/product/Amicon-Ultra-15-Centrifugal-Filter-Units,MM_NF-C7715#documentation*. cited at: 30.11.19].
79. Scientific, T. *https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011307_SupSig_West_Dura_Extend_Dur_Subs_UG.pdf*. cited at: 30.11.19].
80. Häger, W., *Interaction of plant proteins with polygalacturonases from the mustard leaf beetle Phaedon cochleariae*, in *Max Planck Institute for Chemical Ecology*. 2015, Friedrich-Schiller-Universität Jena.
81. Torres, S., et al., *A colorimetric method to quantify endo-polygalacturonase activity*. Enzyme Microb Technol, 2011. **48**(2): p. 123-8.
82. Klockenbusch, C. and J. Kast, *Optimization of formaldehyde cross-linking for protein interaction analysis of non-tagged integrin beta1*. J Biomed Biotechnol, 2010. **2010**: p. 927585.
83. Zhang, L., et al., *Molecular Characterization and Expression Profiles of Polygalacturonase Genes in Apolygus lucorum (Hemiptera: Miridae).* PLoS One, 2015. **10**(5): p. e0126391.
84. Ghedira, R., et al., *The efficiency of Arabidopsis thaliana floral dip transformation is determined not only by the Agrobacterium strain used but also by the physiology and the ecotype of the dipped plant.* Mol Plant Microbe Interact, 2013. **26**(7): p. 823-32.
85. Bent, A., *Arabidopsis thaliana Floral Dip Transformation Method*, in *Agrobacterium Protocols*. 2006. p. 87-104.
86. Rickelmann, K.M.B., C. E., *Effects of Soil Moisture on the Pupation BehaviorofAltica Subplicata(Coleoptera: Chrysomelidae).* The Great Lakes Entomologist, 1991. **24**.
87. Manfredini, C.S., F.; Ferrari, S.; Pontiggia, D.; Salvi, G.; Caprari, C.; Lorito, M.; De Lorenzo, G. , *Polygalacturonase-inhibiting protein 2 of Phaseolus vulgaris inhibits BcPG1, a polygalacturonase of Botrytis cinerea important for pathogenicity, and protects transgenic plants from infection.* Physiological and Molecular Plant Pathology, 2005. **67**(2): p. 108-115.

10 Supplementary Data

Sequences

Bra035741:

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Bra005917:

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Bra005917 + 9nt:

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Bra009238:

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CATGTTCCAGTTCGATATCTCCAAGGTTAAGGTTGCTAAGACAGTTAACCTC
TTGGACTTGAATCACAACGGGATCACAGGGAGTATCCCGGTTCAATGGACA
GAACTTAGTCTCCAGAGTTTCAATGTTAGCTATAATAGATTGTGTGGACCCA
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ACAAGTGTTTGTGTGGTGCACCTCTTCAGAGATGCAAGTGA

Bra005916:

atggataagataacgactacattgctcttttccttgttcgctctcctcctcacggcctctttgtcaAAAGATCTCTGTCA
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CCTACTATCGCCAAGCTCAAGTATCTTCGTTTTCTCTGGCTCAGCTGGACCA
ACCTGACCGGTCCGGTTCCTGAATTTTTGAGTCAGCTCAAGGATCTAGAGTA
CATTAACCTTTCCTTCAATGACCTCTCTGGTTCCATACCCGGTTCTCTCTCTT
TGTTACCTAAACTCGGGATTCTTGAACTAAGCAGGAACAAACTTACAGGTTC
AATACCAGAGTCATTTGGAGCGTTTAAAGGAGTGGTACCTCCTGAGATTTTT
CTATCGCACAACCAGCTATCCGGTTCGATACCAAAATCACTAGGCAACCTC
GATTTTCACCGGATCGATTTCTCCCATAACAAGCTTGAAGGTGATGCTTCGA
TGATGTTTGGAGCCAAAAAGACGTCATGGTCCGTTGATTTATCAAGAAACA
AGCTCCAGTTTGATATTTCCAAGGTTAAAGTGGCTACAACAGTTAATAACTT
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GCTTACTCTTCAGTCTTTCAATGTAAGCTATAACCGACTTTGTGGACGAATA
CCCCAGGGAGGGGACCTTCAGATATTTGATGCTTATGCATATTTACACAACA
AGTGCTTGTGTGGTGCACCTCTTCAGAGTTGCAACGTGGAGATTCAAGCAAC
CGATCTTTATCTAAATTTACCATCAGAATAA

PvPGIP2:

atgtcctcaagcttaagcataattttggtcattcttgtatctttgagcactgcacactcaGAGCTATGCAACCCACA
AGACAAGCAAGCCCTTCTCCAAATCAAGAAAGACCTTGGCAACCCAACCAC
TCTCTCCTCATGGCTTCCAACCACCGACTGTTGCAACAGAACCTGGCTAGGT
GTTTTATGCGACACCGACACCCAAACATATCGCGTCAACAACCTCGACCTCT
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CGCAGCTAAAGTTTCTG
CACAGTTTAAATGTGAGCTTCAACAATCTGTGCGGTGAGATTCCTCAAGGTG
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Agarose Gel/ Western Blots

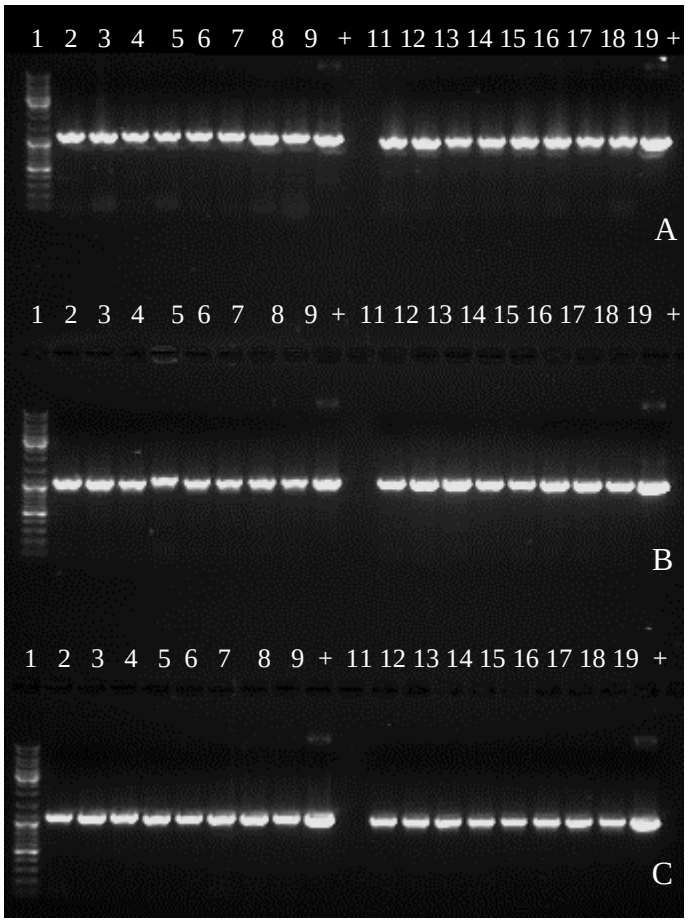

Figure 17: Agarose gel of colony PCR of transformed *A. tumefaciens* colonies. DNA was extracted from eight *A. tumefaciens* colonies, respectively, and amplified with gene-specific primers. Positive controls were amplified from the respective pCAMBIA2300_35SU plasmid (+). A: A2-10: Bra035741 A12-20: Bra005917 B: B2-10: Bra005917 + 9nt B12-20: Bra009238 C2-10: Bra005916 C12-20: PvPGIP2 All tested *A. tumefaciens* clones were positive for their respective insert. A1, B1, C1: GeneRuler DNA Ladder Mix.

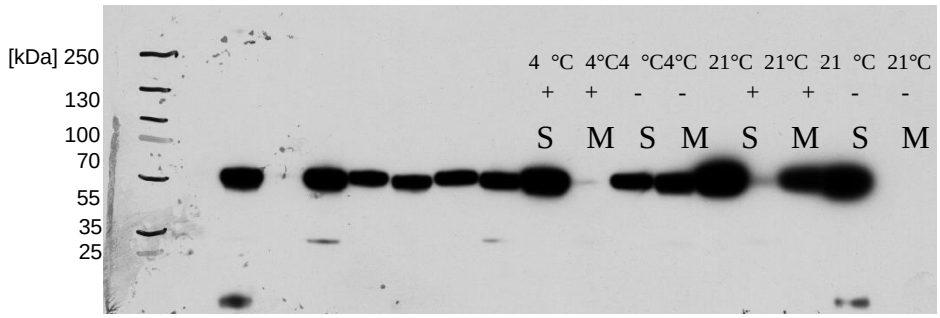

Figure 18: Western Blot test temperature impact on PvPGIP2_GPI with PI-PLC. Membrane preparations from Sf9 cells expressing PvPGIP2_GPI were incubated with sodium acetate buffer at 4 °C or 21 °C. One half of the samples were boiled after mixing with sample buffer (5 min, 95 °C, +). S: supernatant fraction, M: membrane fraction Detection of PGIP_myc with anti-myc antibody, exposure time: 5 min; PageRuler Plus Prestained Protein Ladder was used as size standard. Left samples: irrelevant.

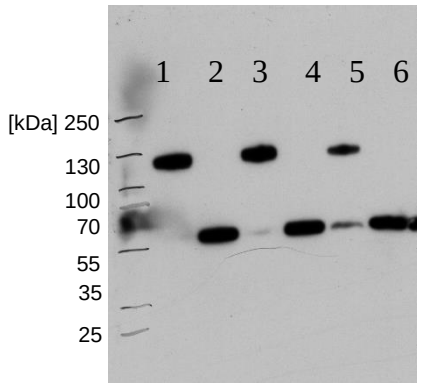

***Figure 19*: Western Blot test culture medium impact on PvPGIP2_GPI with FpPG.** Membrane preparations from Sf9 cells expressing PvPGIP2_GPI were incubated with Fp and several culture media and all controls. Detection of PGIP_myc with anti-myc antibody, exposure time: 5 min; PageRuler Plus Prestained Protein Ladder was used as size standard. 1: PvPGIP2 + FP + FA (ohne Medium), 2: PvPGIP2 + FP (ohne Medium), 3: PvPGIP2 + FP + FA (Sf9 Medium), 4: PvPGIP2 + FP (Sf9 Medium), 5: PvPGIP2 + FP + FA (BMMY Medium), 6: PvPGIP2 + FP (BMMY Medium)

11 Declaration of authorship

I hereby declare that this thesis and the work reported herein was my own work. Information derived from the published and unpublished work of others was acknowledged in the text and references of the bibliography. This thesis has not been submitted in any form for study or examination achievements or another degree at any university.

Place, Date Signature

Ich erkläre, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Die eingereichte Arbeit ist nicht anderweitig als Prüfungsleistung verwendet worden oder in deutscher oder einer anderen Sprache als Veröffentlichung erschienen

Ort, Datum Unterschrift