Studying the Influence of *Arabidopsis thaliana* PGIPs on Performance of *Phaedon cochleariae*

Bachelor-Thesis

vorgelegt an der Ernst-Abbe-Hochschule Jena
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1 Indroduction

On earth there are about 1.5 million species, interestingly about 25 % of these species are assigned to the order of beetles [49]. A categorisation of characteristic features and behaviours lead to various different families and subfamilies. The family of leaf beetles (*Chrysomelidae*) contains about 35 000 species [24]. These herbivorous beetles feed on a variety of host plants, which make some of them an severe pest. On the other hand some species are selective enough in their plant preference to use them for containment of invasive plants or weeds [18].
Phaedon cochleariae, also called the mustard leaf beetle, is one representative of the family of *Chrysomelidae*. It is characterised by its metallic blue to black colour and it can be found in Western Europe, Anterior and Middle Asia and North America. The beetles appear at the end of May and beginning of June in the northwestern and central regions, hatching beetles lay their eggs in August. The lifespan of *P. cochleariae* is on average 33-37 days, containing three larval instars and an eight to ten day pupal stage. Larvae and adults feed on plants of the family *Brassicaceae* preferably near lakes or rivers [8]. Economically, this fact makes *P. cochleariae* a pest for many plants belonging to the cabbage family, like white cabbage, cauliflower, chinese cabbage or broccoli, but also other important crop plants like colza [21]. The primary cell wall of plants is not only responsible for structural and mechanical support, but also to protect the plant from environmental factors like dehydration or invaders. The major polysaccharides present as a complex network in the cell walls are cellulose, hemicellulose and pectin [14]. As a specialist, *P. cochleariae* is able to express digestion enzymes for cell wall degradation, such as cellulases (GH5 and GH45) and polygalacturonases (GH28) [44]. As important biotechnological enzymes, cellulases are able to hydrolyse β (1,4) linkages in cellulose chains[58]. Evidence shows, that cellulases are present in many beetles of the order *Coleoptera*, including for example the xylophagous longhorn beetle or the larvae of the red palm weevil [55], [36]. More focused in this study, polygalacturonases (PGs) are able to hydrolyse the α (1,4) linked galacturonic acid residues and can therefore degrade pectin in the cell walls of plants, resulting in oligogalacturonic acid products with different degrees of polymerisation [1]. For microbial PGs, the induction, in general, is caused by the presence of pectin, polygalacturonic acid and products with a structural similarity to the substrate. A repression can occur because of carbon catabolites, for example glucose, carbon catabolite repressor proteins or even pH level [22]. For *P. cochleariae* many cell wall hydrolysing enzymes were studied, not only outlining the different en-

zymes, but also the different expression times of them in the developmental stages of the organism [27]. The results of further studies show that beetles have received the PGs via horizontal gene transfer from an ascomycete fungus, sharing characteristics of amino acids in microbial and plant PGs [32]. In contrast to the more offensive use of PGs by plant invaders, the plant itself produces PGs, which are involved in cell separation during plant development, which leads to the plant side of this subject.

Arabidopsis thaliana, the model plant used in this thesis is assigned to the family of Brassicaceae. In general the most common regions to find *A. thaliana* are Europe and North America, as well as some coastal areas of Asia and Africa. Even though it has no agricultural importance, it holds many advantages for research, such as a relatively small genome consisting of five chromosomes, a rapid life cycle of about six to eight weeks and, since the year 2000, a completely sequenced genome. In addition various mutant lines of *A. thaliana* are available at Stock Centers [51]. Although *A. thaliana* is no native feeding plant of *P. cochleariae*, the beetles are feeding on it, which makes this plant-insect combination a good model for interaction studies. Looking into plant interaction with insects, especially the ability of plants to defend themselves, are a point of interest.

When it comes to defence, plants use many strategies and mechanisms, for example mechanical defences like thorns, spines, trichomes or idioblasts. Besides these mechanical defence strategies, many plants are able to defend themselves with chemical components. Part of these defence strategy can be toxins, enzymes or other secondary metabolites and proteinaceous inhibitors [10], [42].

Plants can produce a variety of different acting toxins as secondary metabolites, three examples for plant toxins are alkaloids, tannins or poisonous enzymes. Alkaloids are organic structures, containing a heterocyclic ring, which can damage the nervous system or other organs. Common examples for alkaloids are nicotine, taxine or cocaine. Tannins are substances that are able to precipitate proteins and decrease the conversion of food into new body substances [17]. There is a large number of poisonous enzymes, the most common and toxic one is known as ricin. The toxicity is caused by the inhibition of protein synthesis, whereas other effects on apoptosis pathways and the cell membrane were noticed [52], [43]).

There is a wide range of other secondary metabolites, like cyanogenic glycosides and non protein amino acids, to mention just a few of them. Cyanogenic glycosides are derived from an amino acid, and releases cyanide as hydrolysed by enzymes. The formed cyanide can inhibit the cytochrome c oxidase and therefore lead to an insufficient oxygen

origin of PGIP	expression system	reference
tobacco	*Escherichia Coli*	[57]
colza	*Pichia Pastoris*	[4]
rice	procaryotic system	[15]
common bean	wheat	[28]
	tomato	[45]
pear	strawberry	[56]
	persimmon	[53]
	wine grape	[2]
wine grape	tobacco	[41]
bean	tobacco	[11]

Table 1: Examples for PGIP expression in different systems; the first three entries as in vitro expression and from the fifth entry in vivo expression

supply, although plants and animals, including herbivores, are able to detoxify cyanides [9], [6]. Also hydrolysed by a special enzyme, glucosinolates play an important role in plant defence. Myrosinases are able to break down the glucosinolates into various, sometimes toxic products like isothiocyanates. Some specialists, feeding on plants containing these toxic products can detoxify them, or even use these components to locate the host. Mostly deterring against non-specialists herbivores, plants produce non-protein amio acids, like canavanine or mimosine. The effect of these substances can be different, ranging from integration into proteins to toxic effects caused by the breakdown of gut bacteria [6].

Not only enzymes but proteinaceous inhibitors are also produced by plants to defend themselves. For instance proteinase inhibitors produced by plants interact with proteinases used by insects as digestive enzymes [13]. As another proteinaceous inhibitor polygalacturonase inhibitor proteins (PGIPs) can bind to PGs of fungi and herbivorous insects to protect the cell wall of the plant from degradation. The first time PGIPs were mentioned almost 50 years ago, in 1971 [3]. Since then PGIPs have been characterised, studied and expressed in different systems (see table 1) and no plant species with a complete absence of PGIPs were found [31]. Studying the mechanism of inhibition, it was discovered that not all PGIPs are able to recognise all PGs [5], which cause an arms race between pathogens or phytophages and plants. In this context, most plants possess more than one PGIP gene with different recognition abilities, for example the common bean possesses four PGIPs, wheat has three PGIPs and *Arabidopsis thaliana* owns two PGIP genes [33]. The use of different signalling pathways underlined the studies in 1999 in which a difference in wound signalling pathways of plant PGIPs and a more com-

plex regulation of damage response genes were hypothesised [47]. The exact interaction of PGs and PGIPs remains unknown, although it is assumed that the mechanism of inhibition may be competitive, non-competitive or mixed, depending on the PG-PGIP combination [5], [31]. Until today many PG-PGIPs systems were studied, in beetles actually less than for fungal infections: The inhibition of PGs from *Aspergillus niger* and *Fusarium moniliforme* in combination with PGIPs of pearl millet or a pear fruit PGIP expressing tomato plant in combination with *Botrytis cinerea* PGs, together with numerous other systems were already investigated [46], [45]. In addition to that, PGIP encoding genes are candidate genes for resistance for bruchid resistance in mungbean [16]. The recognition of PG by PGIPs is proven to be associated with the residues, for example changing the PG residue acted as a switch for *Phaseolus vulgaris* PGIP to recognise the PG of *Fusarium verticillioides* [5]. The identity of the PGIPs occurring in the same plant are relatively high: The cDNA of two PGIPs extracted from soy bean roots share an identity of 92 %, at amino acid level there is an 88 % identity between soy bean and common bean PGIPs [37]. *A. thaliana* PGIPs share an identity of 80 %, comparing them with the PGIPs of *Brassica napus*, the AtPGIP 1 is with an 81,8 % identity more related to BnPGIP 2, whereas the AtPGIP 2 is more related to the BnPGIP 1 (see figure 2) [25], [35].

The expression of the AtPGIPs was measured in the leaves, petioles, stems and roots, mostly present in the epidermis, vascular bundle and vascular cylinder [20]. PGIPs are in most cases up regulated by environmental stress stimuli, including low temperatures, wounding of plant tissue and feeding or invasion of pathogens and herbivores[33], [25], [35]). Bringing the organisation and the function of these proteins together, it supports the idea of tandem duplications of stress related genes as an advantage for survival of plants in challenging surroundings [31].

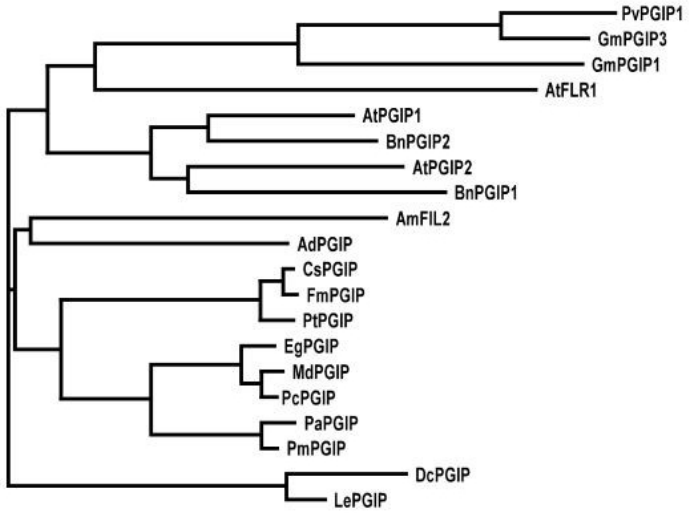


Figure 2: Phylogenetic relationship between various plant PGIPs. The proteins used for alignment were as follows: MdPGIP (Malus domestica, U77041); PcPGIP (Pyrus communis, L09264); EgPGIP (Eucalyptus grandis, AF159167); PaPGIP (Prunus armeniaca, AF020785); PmPGIP (Prunus mahaleb, AF263465); CsPGIP (Citrus sinensis, Y08618); FmPGIP (Fortunella margarita, AB020529); PtPGIP (Poncirus trifoliata, AB020528); DcPGIP (Daucus carota, AY081214); LePGIP (Lycopersicon esculentum, L26529); AmFIL2 (Antirrhinum majus, X76995); AdPGIP (Actinidia deliciosa, Z49043); BnPGIP2 (Brassica napus); AtPGIP1 (Arabidopsis thaliana, AF229249); AtPGIP2 (A. thaliana, AF229250); BnPGIP1 (B. napus); PvPGIP1 (Phaseolus vulgaris, X64769); GmPGIP3 (Glycine max, AF130974); GmPGIP1 (G. max, X78274); AtFLR1 (A. thaliana, AF136588); cited from [35]

In addition to the inhibitory activity, PGIPs favour the accumulation of oligogalacturonidess (OGs), which, in the right chain length of 10-14 sugars, can act as elicitors for other plant defence responses [25], [19]. Other known elicitors for a variety of plant defence responses are salicylic acid, jasmonic acid and ethylene. With *A. thaliana* as studied organism, the effect of these three different signalling molecules on the regulation of PGIPs was investigated. The results show that AtPGIP 1 expression is induced by OGs and *Arabidopsis thalilana* polygalacturonaseinhibitor protein (AtPGIP) 2 is mediated by jasmonic acid, the expression of both PGIPs is independent from salicylic acid and ethylene [25]. These examples show only a small insight in plant defence responses and regulations. However the focus of this thesis will be the PG-PGIP interaction of herbivores and plants.

2 Purpose of this bachelor thesis

The interaction of polygalacturonases in herbivouros insects and their counterparts, the polygalacturonase-inhibiting proteins, which can be found in plants, is not studied in detail. To enhance the knowledge of this interaction and possible inhibition, it is essential to study the influence of these proteins from different origins on herbivouros insect life history. For this reason, this bachelor thesis targets the polygalacturonases found in *Phaedon cochleariae* in combination with the two polygalacturonase inhibiting proteins found in *Arabidopsis thaliana*. A major aspect studied here, is the influence of the inhibitors on the growth, more specific, the weight gain of *Phaedon cochleariae*. In addition to that, an expression of both PGIPs of *Arabidopsis thaliana* bound to V5-beads, is a target. A successful expression would create the foundation for interaction studies like binding assays, which could determine an inhibition of this PG-PGIP constellation. The results of this study could be applied on economical important crop plants, to investigate further into plant protection and plant defence against pests.

3 Materials and Equipment

3.1 Materials

Eppendorf Conical Tubes 50 ml	Eppendorf AG, Hamburg
CELLSTAR CELLreactor™ Tubes 50 ml	Greiner bio one, Frickenhausen
Centrifuge Tubes with screw caps, 50 ml	Labcon, Hannover
Reaction Tubes 1.5 ml/2 ml, PP, graduated	Greiner bio one, Frickenhausen
96-Well PCR Plate, Non-Skirted	STARLAB International GmbH, Hamburg
PCR 8er-Softstrips 0.2 ml,	Scientific GmbH, Oldendorf
6-/24 Well CytoOne® Platte, unbehandelt	STARLAB GmbH, Hamburg
Ambion Nuclease-Free Water	Thermo Fisher Scientific GmbH, Bonn
LiChrosolv® Water for chromatography	Merck KGaA, 64271 Darmstadt
Ethanol Rothipuran ≥98 %	Carl Roth GmbH & Co., Karlsruhe
2-Propanol Rothipuran ≥98 %	Carl Roth GmbH & Co., Karlsruhe
SDS ≥99.5%, Blotting-Grade	Carl Roth GmbH & Co., Karlsruhe
Ampicillin sodium salt, CELLPURE® ≥91 %	Carl Roth GmbH & Co., Karlsruhe
Gibco™Gentamicin (50 mg/mL)	Thermo Fisher Scientific GmbH, Bonn
Tris base (≥99.9 %)	Sigma-Aldrich Chemie GmbH, München
hydrochloric acid 37%	Carl Roth GmbH & Co., Karlsruhe
EDTA (0.5 M), pH 8.0	invitrogen™, Thermo Fisher Scientific GmbH, Bonn
ethidium bromide 1 % (10 mg/ml)	Carl Roth GmbH & Co., Karlsruhe
Rothiphorese® 50x TAE Buffer	Carl Roth GmbH & Co., Karlsruhe
SeaKem® LE Agarose	Lonza Group AG, Basel
6x Orange DNA Loading dye	Thermo Fisher Scientific GmbH, Bonn
O´GeneRuler DNA Ladder Mix	Thermo Fisher Scientific GmbH, Bonn
Stahlkugeln 3/32”	ASK Kugellagerfabrik Artur Seyfert
ColiRollers Plating beads	EMD Millipore Corp., Billerica, MA, USA
Quick load *Taq* 2x Master Mix	New England Biolabs Inc., Ipswich, Massachusetts
Q5® High Fildelity 2x Master Mix	New England Biolabs Inc., Ipswich, Massachusetts
AccuPrime™ Taq DNA Polymerase, high fildelity, invitrogen™	Thermo Fisher Scientific GmbH, Bonn
AccuPrime™ PCR Buffer I/II, invitrogen™	Thermo Fisher Scientific GmbH, Bonn
pIB/V5 His TOPO™ TA Expression Kit	Thermo Fisher Scientific GmbH, Bonn
pMIB/V5-His A, B and C Vector Kit, invitrogen™	Thermo Fisher Scientific GmbH, Bonn
Not I	New England Biolabs GmbH, Frankfurt am Main
Kpn I, recombinant	New England Biolabs GmbH, Frankfurt am Main
T4 DNA Ligase	New England Biolabs GmbH, Frankfurt am Main
Goat Anti-V5, agarose immobolized	Biomol GmbH, 22525 Hamburg
SOC Media	Amresco Inc., Solon, Ohio
Sf-900™ III SFM	Gibco™, Thermo Fisher Scientific GmbH, Bonn
Sf9 cells in Sf-900™ II SFM	Gibco™, Thermo Fisher Scientific GmbH, Bonn

FuGENE® Transfection Reagent	Promega GmbH, Mannheim
V5 Tag Monoclonal Antibody (E10/V4RR)	invitrogen™, Thermo Fisher Scientific GmbH, Bonn
Milchpulver Blotting-Grade, pulv., low fat	Carl Roth GmbH & Co., Karlsruhe
4–12% Criterion™XT Bis-Tris Protein Gel, 18 well, 30 µl	Bio-Rad Laboratories GmbH, München
4–12% Criterion™XT Bis-Tris Protein Gel, 26 well, 15 µl	Bio-Rad Laboratories GmbH, München
20x XT MOPS Running Buffer Kit	Bio-Rad Laboratories GmbH, München
XT Sample Buffer	Bio-Rad Laboratories GmbH, München
XT Reducing Agent	Bio-Rad Laboratories GmbH, München
PERDROGEN® (Wasserstoffperoxid) 30 Gew.%	Riedel-de Haen GmbH, Seelze
Luminol for chemiluminescence	Fluka, Sigma-Aldrich Chemie GmbH, München
p-Coumaric acid	Sigma-Aldrich Chemie GmbH, München
Carestream® Kodak® autoradiography GBX fixer/replenisher	Sigma-Aldrich Chemie GmbH, München
Carestream® Kodak® autoradiography GBX developer/replenisher	Sigma-Aldrich Chemie GmbH, München
Thermo Scientific™ Gene JET Plasmid Miniprep Kit	Thermo Fisher Scientific GmbH, Bonn
Invitrogen™ PureLink™ HiPure Plasmid Thermo Scientific™ GeneJET Plasmid Midiprep Kit	Thermo Fisher Scientific GmbH, Bonn
DNA Clean & Concentrator™-5	Zymo Research, Irvine, CA, USA
Zymoclean™ Gel DNA Recovery Kit	Zymo Research, Irvine, CA, USA

3.2 Equipment

Eppendorf Research Pipette Eppendorf Research plus Pipette	Eppendorf AG, Hamburg
QikSpin	Laborbedarf Süd, München
myFUGE Mini	Benchmark Scientific Inc., Sayreville, NJ, USA
Centrifuge 5424 R/ 5415 R/ 5810 R	Eppendorf AG, Hamburg
Avanti™ J-20 XP Centrifuge	Beckman Coulter GmbH, Krefeld
Vortex Genie 2	Scientific Industries, NY, USA
Thermomixer comfort	Eppendorf AG, Hamburg
ICE Maker	Hoshisaki, Willich-Münchheide
Laboratory Refrigerator Type Special 489 with forced air cooling	Phillip Kirsch GmbH, Willstätt-Sand
water bath	Dinkelberg analytics GmbH, Gablingen
Certomat IS	Satorius AG, Göttingen
MC1000 HE-EVD climate chamber	Snijders scientific, Tilburg, NL
EB2E climate chamber	Snijders scientific, Tilburg, NL
Mastercycler epgradient S	Eppendorf AG, Hamburg
Nanophotometer N60	Implen GmbH, München

TissueLyser LT	QIAGEN, Hilden
Subcell GT (wide mini)	Bio-Rad Laboratories GmbH, München
Biometra P20 Minicell Power Pack	Analytik Jena AG, Jena
Criterion™Cell	Bio-Rad Laboratories GmbH, München
PowerPac™ Universal Power Supply	Bio-Rad Laboratories GmbH, München
Immun-Blot® PVDF Membranes for Protein Blotting	Bio-Rad Laboratories GmbH, München
Criterion™blotter Filter paper	Bio-Rad Laboratories GmbH, München
Stuart rotator SB 3	Cole-Parmer Ldt, Staffordshire
Stuart Shaker SSM3	Cole-Parmer Ldt, Staffordshire
Dark Reader® DR196 Transilluminator	Clare Chemical Research Inc., Dolores, CO
GeneGenius Bioimaging System Syngene Adventurer Pro	Syngene, Cambridge, UK
3730XL DNA Analyser	Hitachi, Applied Biosystems

3.3 Software

SigmaPlot 11.0	Systat Software, Inc., Erkrath
ImageJ	open access
Microsoft Excel	Microsoft Corporation, Washington
GIMP 2	open access
SeqManPro 15	DNASSTAR, Inc.
NanoPhotometer® N 60	Implen GmbH, München
Adobe Illustrator CS5	open access

4 Methods

4.1 Plant growth

The plants used in the assays were the *Arabidopsis thaliana* Columbia wild type (WT) and two mutants, where one PGIP is knocked out respectively. The seeds were purchased from the *Nottingham Arabidopsis Stock Centre* (mutant lines: PGIP 1 mutant: GK_092G09, PGIP 2 mutant: GK_717A02) and seeded together with *Steinernema feltiae* in the green house of the institute. They remain 4 days in a fridge before they were grown in the short-day climate chamber with 21 °C, a humidity of 40-50 % and a light exposure of ten hours a day. The plants were used after 5 weeks.

4.2 Beetle rearing

The first generation of *Phaedon cochleariae* was collected near the city of Bayreuth (Germany). The continuous culture was reared in the laboratory, on leaves of *Brassica rapa chinensis*. They were kept at 20 °C with a light cycle of 16 hours light and 8 h darkness.

4.3 Feeding assays

4.3.1 Feeding assay on hole plants

The small plants were covered with plastic cups where the bottom was cut off. They grow into the cups and when the beetles were placed on them, each plant was covered with a nylon ankle sock. For the feeding assay 40 replicates per plant line (wt, PGIP 1 m, PGIP 2 m) were used and three neonates were placed on the plant. The neonates were weighted in a pool of five. After nine days, the larvae were weighted separately. The larvae of six plants were collected, all three larvae of a plant in one 1.5 ml reaction tubes and frozen in liquid nitrogen. All samples were stored at -80 °C. The remaining larvae of 27 plants were raised to adulthood, the date of hatching was documented and they were collected. For the analysis of the weight differences an ANOVA on Ranks was performed.

4.3.2 Feeding assay on plant discs

For the plant disc assays 6-Well Plates were prepared per line (WT, PGIP 1 m, PGIP 2 m). A filter paper with 50 µl distilled water and an *Arabidopsis thaliana* plant disc with 14 mm diameter was placed in each Well. The first plant disc assay was performed with ten 6-Well plates and second instar larvae of *P.chochleariae* for 18 hours at 20℃. The second assay was performed with eight 6-Well plates per line and started with neonates of *P.chochleariae*. Until pupation the plant discs were replaced daily. To keep the plant discs in a good condition over night the filter paper was kept moist. The discs were photographed each day and the remaining leaf area was calculated with the software ImageJ. In addition every instar of the larvae was documented, they were weighted after 12 days to determine the weight gain. For all data analyses an ANOVA on Ranks was performed.

4.4 Extraction of genomic DNA

The method of genomic DNA (gDNA) isolation is based on the description by Edwards et al. [23]. A plant disc, sized like the lid of a reaction tube, was harvested from a plant. Three metal beads were added to the tube and it was placed in liquid nitrogen. The frozen samples were placed in the TissueLyser LT, shaken for 1 min at 50 Hz and centrifuged down for 1 min at full speed. 400 µl of extraction buffer was added and the sample was vortexed for 5 sec afterwards. The debris was centrifuged down for 1 min at full speed. All supernatant was transferred into a new 1.5 ml reaction tube. Further, 300 µl of isopropanol was added and the sample was mixed by inverting 5 to 10 times. After 5 min incubation, the samples were centrifuged 5 min at full speed to pellet the DNA. All supernatant was removed and the sample was incubated at 37 ℃for a few minutes to dry the pellet. At the end the pellet was re-dissolved in 100 µl water by shaking gently.

4.5 Genotyping PCR for *Arabidopsis thaliana*

To assure that the mutant lines had the right gene knockout, gDNA from the wild type and the mutant lines were tested via genotyping PCR (tables3, 4). This method includes two separate PCRs with two different primer combinations respectively (table 2). The first primer set binds gene specific, the second one binds in the inserted transfer DNA (tDNA). After running the PCR the results were analyzed by agarose gel electrophoresis. To assure the results another PCR with the same template and the self designed primer

for the amplification of the insert for pMIB vector was performed with 35 cycles. (See table 6, 5)

4.6 Gel electrophoresis

If not stated otherwise, all electrophoreses were performed with 1.2 % agarose gels. For the smaller gels the voltage of 160 V was used for 20 to 30 min, the larger ones were run with 160 to 200 V for 20 to 40 min. All samples were dyed with the 6x Orange DNA Loading dye. The DNA was stained with ethidium bromide and a picture was taken under UV light. If bands needed to be cut out of the gel, this was realized before the picture was taken.

4.7 Cloning PGIP 1 and PGIP 2 plasmids in TOP 10 *Escherichia coli* (*E.coli*)

4.7.1 Amplification of the insert

The amplification was done for each vector respectively, for the pIB vector some optimizations were made to improve the amplification. The ubiquitin C gene (UBC) , a housekeeping gene of *Arabidopsis thaliana* was also amplified in the same PCR to assure that the concentration in the template is high enough. Template for the amplification was a cDNA pool, extracted from wild type *Arabidopsis thaliana* plant material via RNA extraction and reverse transcription. After both amplifications the insert was cleaned with the DNA Clean & Concentrator™-5. The elution step was done with water instead of the elution buffer, all remaining steps were done like described in the manufacturers manual.

4.7.1.1 Conditions for the pIB vector

To maximise the result of the amplification of the insert for the pIB vector some optimisations were required. The conditions before and after the optimisation can be found in tables 7 and 8 .

4.7.1.2 Conditions for the pMIB vector

The conditions for the amplification of the insert for the pMIB vector can be found in tables 9 and 10 . The PCR was performed with 35 cycles.

4.7.2 Restriction digest of pMIB insert and vector

The insert and the vector were cut with the restriction enzymes Not I and Kpn I. With the vector the enzymes were tested each respectively to ensure the activity (tables 11, 12). After restriction of the inserts, they were cleaned up with the DNA Clean & Concentrator-™-5, as described before the elution was done with water instead of the elution buffer, all remaining steps were done like described in the manufacturers manual. The vector was load on an agarose gel and the bands were cut out on a Transilluminator table. It was cleaned up with the Zymoclean Gel DNA Recovery Kit. All steps were done like described in the manufacturers manual, accept of the elution step, that was performed with water.

4.7.3 Ligation and Insertion

4.7.4 Insertion for pIB vector construct

For the pIB/V5 His TOPO™ TA Expression Kit no ligase is needed. About 10 ng insert were used, the incubation time were 30 min at room temperature (table 13).

4.7.5 Ligation for pMIB vector construct

The ligation for the pMIB vector was done with about 60 ng vector and 50 ng insert for 1 hour at room temperature. After this step the ligase was inactivated at 65 °C for 10 min (table 14).

4.7.6 Transformation with TOP 10 *E.coli* for pIB an pMIB vector

The transformation was done with 3 µl plasmid respectively, for the pIB vector with 20 µl and for the pMIB with 25 µl of TOP 10 *E.coli*. All samples were treated with a 30 sec heat shock and 2 min incubation on ice. Subsequently 250 µl of SOC medium was added and the cultures were incubated for 1 h at 37 °C.

4.7.7 Plating the colonies

With the help of the ColiRollers 150 µl and 50 µl of each sample were plated on LB medium agar plates with ampicillin. The plates were incubated at 37 °C over night and after that stored in the refrigerator at 4 °C.

4.8 Colony PCR

Before sequencing the samples can be preselected via colony PCR. For the pIB vector construct the self-designed pIB forward primer for each PGIP and the OpiE2 Reverse Sequencing Primer, delivered with the pIB/V5 His TOPO™ TA Expression Kit were used to proof the presence of the insert with the right orientation in a colony. The primers for the pMIB vector construct only need to assure the presence of the right insert, because of the restriction digest, for this reason the OpiE2 Forward and Reverse Primer were used. The template for the PCR were colonies from the transformed *E.coli* plates (4.7). A Master mix containing the Quick load *Taq* 2x Master Mix, a primer mix and water was prepared in a PCR eight reaction Softstrip (table 15). A colony was picked with a pipette tip and transferred into one of the prepared reaction tubes. After this transfer the rest of the colony was transferred in 10 µl of LB medium. The PCR was performed with 35 cycles (table 16). The PCR products were load on a gel and the bands with the right size of nearly 1000 bp were selected for sequencing. To get more candidate colonies the colony PCR was done twice.

4.9 Sequencing

For sequencing a 96-Well plate was prepared with the samples. The Opi forward and reverse primer of the respective vector was used.

4.10 Mini Plasmid preparation

For the plasmid preparation the 10 µl back up of the colony PCR was transferred in 4 ml LB medium an incubated as shake culture over night at 37°C. 2 ml of this overnight culture was ere used for the preparation with the Thermo Scientific™ Gene JET Plasmid Miniprep Kit. The steps of the manufacturers manual were improved. The cell debris and the chormosomal DNA were centrifuged for 10 min at 16000 xg. For binding and elution the columns were centrifuged with 12000 xg, the washing step was performed with 16000 xg. To remove the residual ethanol of the washing buffer the columns were centrifuged for 1 min with maximum speed. All remaining steps were performed as described in the manufacturers protocol.

4.11 Transfection and harvesting of Sf9 cells

The transfection was done with FuGENE® Transfection Reagent from Promega for either a 24- or a 6-well plate. All steps of the transfection were done under laminar flow cabinet. The cells were taken from a permanent, adherent culture that was grown in a 6-/24-Well plate on Sf-900™ III SFM until the confluence reached about 70 %. First a mixture of plasmid, FuGENE® Transfection Reagent and culture medium was prepared and incubated for 10 min at room temperature (table 17). The present culture medium from the insect cell plates was removed and replaced by 475 µl for a 24- well plate and 1900 µl for a 6-well plate. From the prepared plasmid- FuGENE® mixture, 25 µl were dropped into the center of a 24-well plate, 100 µl to a 6-well plate. The plate was shook horizontally and incubated for 72 hours at 37 °C. After incubation the medium was collected in 1.5 ml reaction tubes and centrifuged for 5 min at 4 °C, maximum speed. The supernatant was collected and stored at 4 °C.

4.12 Midi plasmid preparation

The Midi preparation was realised with the Thermo Scientific™ GeneJET Plasmid Midiprep Kit. All steps were done as described in the manufacturers manual, just the last resolubsilisation step was performed with water.

4.13 Incubation and binding of the PGIPs to V5 beads

15 µl V5 beads were equilibrated by adding 150 µl culture medium, vortexing and centrifuging with 1000 xg for 2 min at 4 °C. The supernatant was removed and the equilibration was repeated two times.
From the 24-Well plate transfection (Transfection and harvesting of Sf9 cells) the whole sample was added to the 15 µl V5 beads, 1000 µl were added form the 6- Well transfection. The samples were incubated over night at 4 °C on a rotating platform. The samples were centrifuged with 1000 g for 2 min at 4 °C and the supernatant was kept as unbound fraction for Western Blot. 150 textmu l of washing detergent consisting of 10 mM Tris HCl pH 7.5, 150 nM NaCl and 0.5 nM EDTA were added to the sample, mixed by vortexing and centrifuged. The washing step was repeated one time with the washing detergent and one time with Merck water. Both washing fractions were kept for Western Blot.

4.14 SDS-Page

For the SDS-PAGE the three samples of PGIP 2 for the pIB and the pMIB vector were applied pooled and separately. 6 µl of sample were prepared with reducing agent, sample buffer and SDS (table 18). All samples were heated up to 95 °C for 5 min and then stored on ice. All 10µl sample and 5µl marker were transferred to a SDS gel. The SDS PAGE ware performed with 500 ml MOPS buffer at 130 volt (V) for 1.5 h.

4.15 Western Blot

To prepare the Western Blot the fibre pads, the filter papers and the Gel were soaked in transfer buffer (Tris-Glycine buffer, 10% methanol). The PVDF membrane was rehydrated with 100% methanol, washed with distilled water and also soaked in transfer buffer. A sandwich consisting of fibre pads on the outside, the filter papers next and the gel in direct contact with the PVDF membrane in the centre was prepared and transferred into the blotting tank. The Western Blot was performed using transfer buffer with 100 V for 30 min. All following steps were performed with agitation. After the run the membrane was washed with distilled water and blocked with 1x TBS, 0,1 % Tween and 20.5 % non fat dry milk for one hour. Over night the membrane was incubated with 1x TBS, 0,1 % tween and 20.5 % non fat dry milk and 1:10000 anti-V5 antibody. Next, the membrane was washed three times for 10 min with 1x TBS and 0,1 % Tween 20. One last washing step was performed with 1x TBS for 5 min. The remaining TBS was removed and the ECL solution (table 19) was added for 1 min. In a dark room a film was exposed to the membrane and developed.

5 Results

5.1 Genotyping

The genotyping PCRs were performed as described in chapter see 4.5 to prove the knockouts in the PGIP mutant plants. The first, gene specific primer combination helps to distinguish between a WT and a heterozygous plant. The second t-DNA specific primer combination helps to distinguish between homo- and heterozygous plants. The agarose gel of the PCR results were analysed and the length (table 2) and presence of the band were correct except of the light bands for the PGIP 1 mutant with the primer for the T-DNA specific PGIP 2 gene [see Figure 3]. Hence, another PCR with the same template and the self designed primer for the amplification of the insert for pMIB vector was performed and analysed [see Figure 4]. With the results of the second PCR the right knockout in the mutant lines were proved.

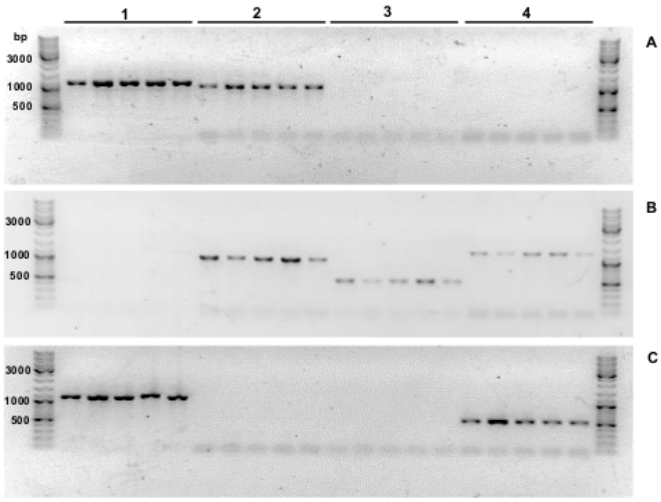


Figure 3: First Genotyping PCR: 1) primer PCR 1 PGIP 1; 2) primer PCR 1 PGIP 2; 3) primer PCR 2 PGIP 1; 4) primer PCR 2 PGIP 2; A) template WT plant gDNA; B) template PGIP 1 mutant plant gDNA; C) template PGIP 2 mutant plant gDNA

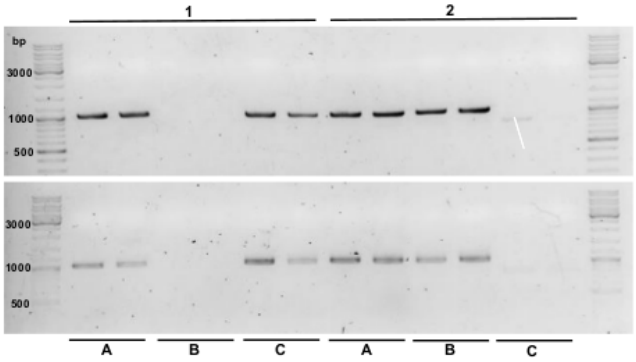


Figure 4: Genotyping PCR with self designed primers: 1) primer PGIP 1 self designed; 2) primer PGIP 2 self designed; A) template WT plant gDNA; B) template PGIP 1 mutant plant gDNA; C) template PGIP 2 mutant plant gDNA

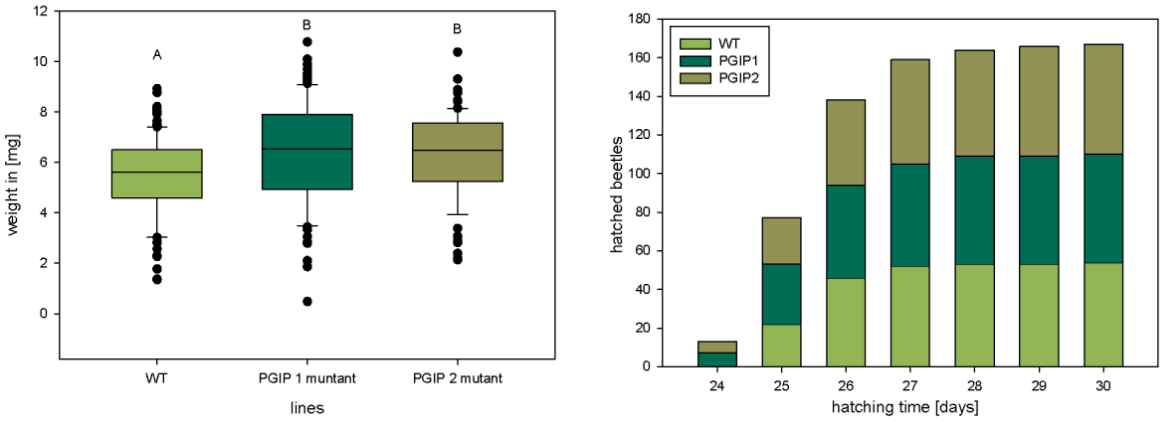
5.2 Feeding assays

5.2.1 Feeding assay on hole plants

With the hole plant feeding assay it was attempted to determine the influence of the AtPGIPs on performance (body mass, hatching time, reproduction) of *P. cochleariae*. This assay was performed with three larvae on a hole plant. One day after hatching the larvae were placed on the plants and the plants stayed at the climate chamber until the larvae reached the third instar. A part of the third instar larvae were weighted, the weight gain of all three lines was determined. The data of all larvae per line were plotted in a diagram [see Figure 5a].

The result was that there is a significant difference in weight gain between the WT and the two PGIP mutant lines, whereas there is no significant difference between the PGIP 1 and PGIP 2 mutant lines. According to this results the weight gain was influenced by the PGIPs in comparison to the WT.

The remaining larvae were raised to adulthood and when the beetles were hatched the dates were compared and displayed in a diagram [see Figure 5b]. No significant difference between the hatching times of the three lines were determined. After 30 days all remaining pupae were assumed as dead. The collected beetles were fed with *Brassica rapa chinensis* until they laid eggs. By observing the beetles and the second generation of larvae the fitness in form of size, feeding behaviour and reproduction of the beetles was determined. There was no difference between the fitness of the beetles fed as larvae on the mutants and the beetles fed as larvae on the WT.



(a) Feeding assay: Larval weight of the beetles feeding on three *Arabidospis thaliana* lines (P=0.05)

(b) Feeding assay: Hatching times of the beetles feeding on three *Arabidospis thaliana* lines

Figure 5: Feeding assay: weight gain and hatching times

5.2.2 Feeding assay on plant discs

The plant disc assays were performed to define the amount of fed leaf material by *P. cochleariae* over a certain time span. For all plant disc assays the plant discs were photographed and changed every day until the end of the assay. Over a longer period the values of the fed area of each replicate were added to calculate the total fed area over the time. The colour channels of plant disc photographs were separated and the channel with the most contrast was converted into a black and white picture. With help of the software the remaining leaf area was calculated for each replicate. The difference between the 14 mm plant disc and the remaining plant disc area was determined and with the help of Sigma Plot 11, the results were presented.

5.2.2.1 First plant disc assay 18 hours

As described further the second instar larvae were placed in the 6-Well plates, together with the plant disc. After 18 h the plant discs were photographed and analysed. Between the wild type and the PGIP 1 mutant was no significant difference in the fed area, however there is a significance in the difference between wild type and PGIP 1 compared to the PGIP 2 mutant line. Hence, the PGIP 2 mutant line fed less leave material than the WT or the PGIP 1 mutant line [see Figure 6].

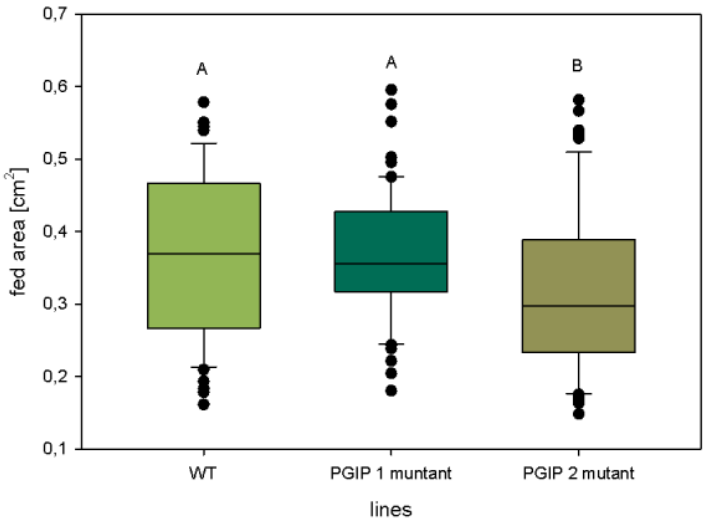


Figure 6: Plant disc assay 18 h: Fed area from *P.cochleariae* on three *Arabidospis thaliana* lines over 18 hours (P=0.05)

5.2.2.2 Second plant disc assay from neonates to adulti

At the beginning of the assay the neonates were placed in the 6-Well plates with the plant discs. The disc was photographed and changed daily and the instars of the replicates were recorded until the pupae hatched. With a mortality rate of over 60%, from 48 replicates in the beginning of the assay only 19 wild type, 16 PGIP 1 mutant line and 13 PGIP 2 mutant line larvae survived until day 12 of the assay. The fed area of these replicates were analysed. A quotient between the gained weight and the fed area was formed and analysed. A One Way ANOVA proved, that there is no significant difference between the gained weight [see Figure 8b], the fed area [see Figure 8a] or the quotient of these two [see Figure 9] within the three lines. These results may be interpreted with caution due to the small number of replicates, an assay with more replicates could come to a different conclusion.

The times of the different instars of the beetles were documented and displayed. There is no extension, reduction or delay of instars between the beetles feeding on the three different *Arabidospis thaliana* lines [see Figure 7].

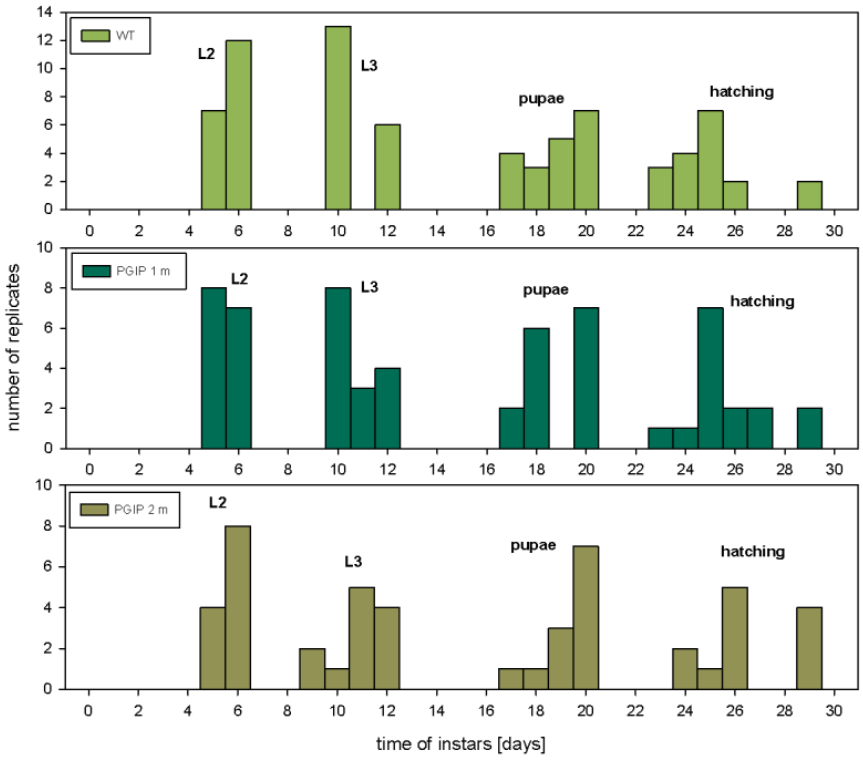
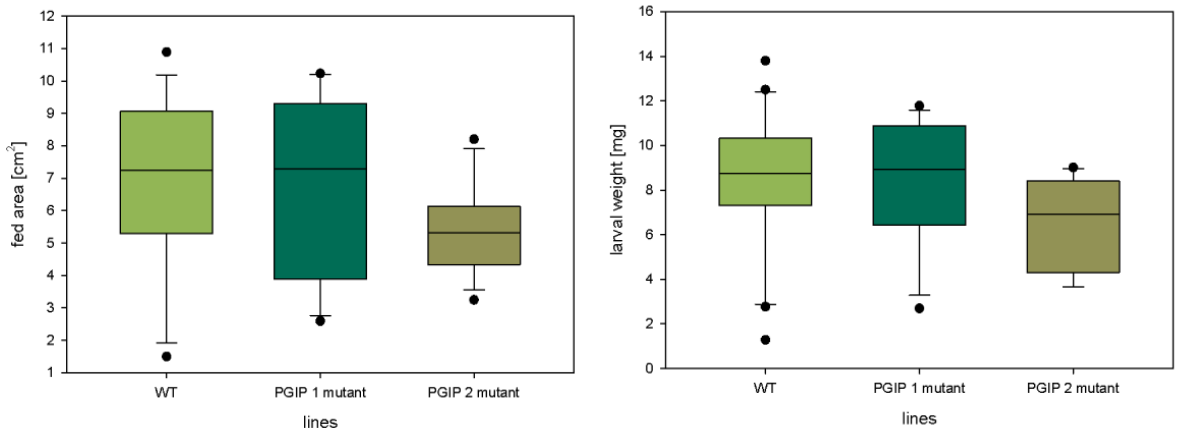


Figure 7: Plant disc assay from neonate to adulti: Time of different instars of *P.cochleariae* feeding on the three *Arabidospis thaliana* lines



(a) plant disc assay from neonate to adulti: fed leaf area from beginning until the end of the assay (P=0.05)

(b) plant disc assay from neonate to adulti: weight of the larvae in third instar, before pupating (P=0.05)

Figure 8: plant disc assay from neonate to adulti: fed leave area and larval weight

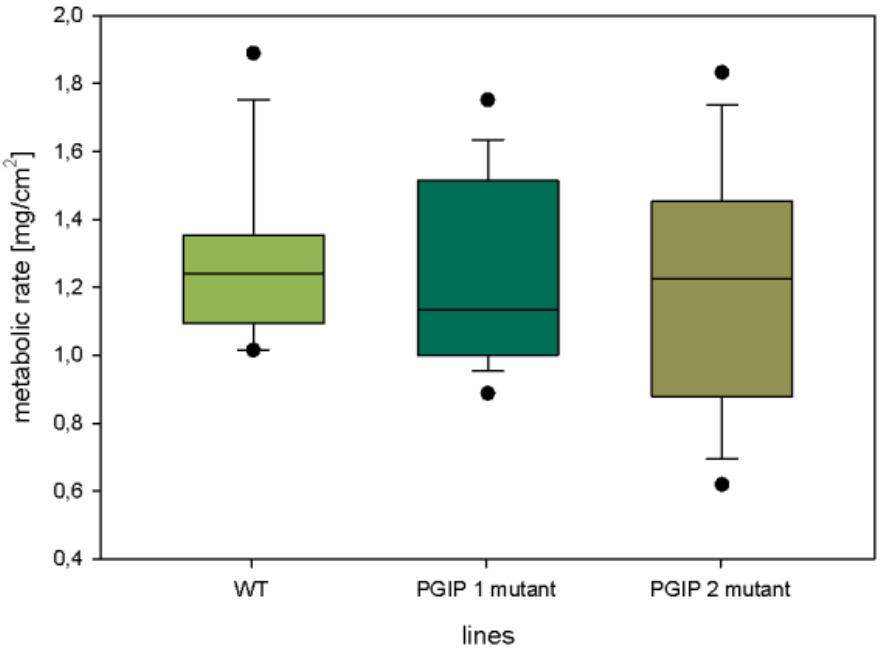


Figure 9: Plant disc assay from neonate to adulti: metabolic rate of beetles feeding on the three *Arabidospis thaliana* lines in mg gained weight per fed cm^2 leaf area (P=0.05)

5.3 Construction of the plasmids for expression in insect cells

For the expression of the AtPGIPs,in insect cells, the plasmids were amplified in *E.coli*, using two different vectors. The difference was in the signal peptide of the vectors, the pMIB vector contains a honeybee melittin secretion signal, the pIB vector was used with the native signal peptide of the PGIPs, for which a better expression is expected. The two signals were tested to determine if the recognition of the signal peptide in insect cells works better with an insect secretion signal or the native plant secretion signal. To identify the best method, plasmids with both vectors were created for each PGIP respectively. For the pMIB vector the multiple cloning site B was used, the primer were designed to fit the reading frame of the signal peptide and the V5 epitope for a later binding to the V5 beads. The pMIB insert was amplified without the native signal peptide. The primer combination for pIB vector were designed starting with the Kozak translation initiation sequence and an ATG start codon, as described in the pIB vector manual, additionally the V5 epitope was designed to be in reading frame. In Addition, for the amplification of the inserts, the UBC housekeeping gene of *Arabidopsis thaliana* was amplified and transferred on the agarose gel as positive control for the integrity of

the template. The length of the PGIPs are about 1000 bp, whereas the UBC has a length of 197 bp. All PCR products with desired length were determined on the gel [see Figure 10]. The pMIB vector was cut and transferred on a gel, the desired bands were cut and the vector was cleaned with the described Kit [see Figure 11]. After the transformed *E.coli* were plated and incubated, enough candidate colonies for a colony PCR were present.

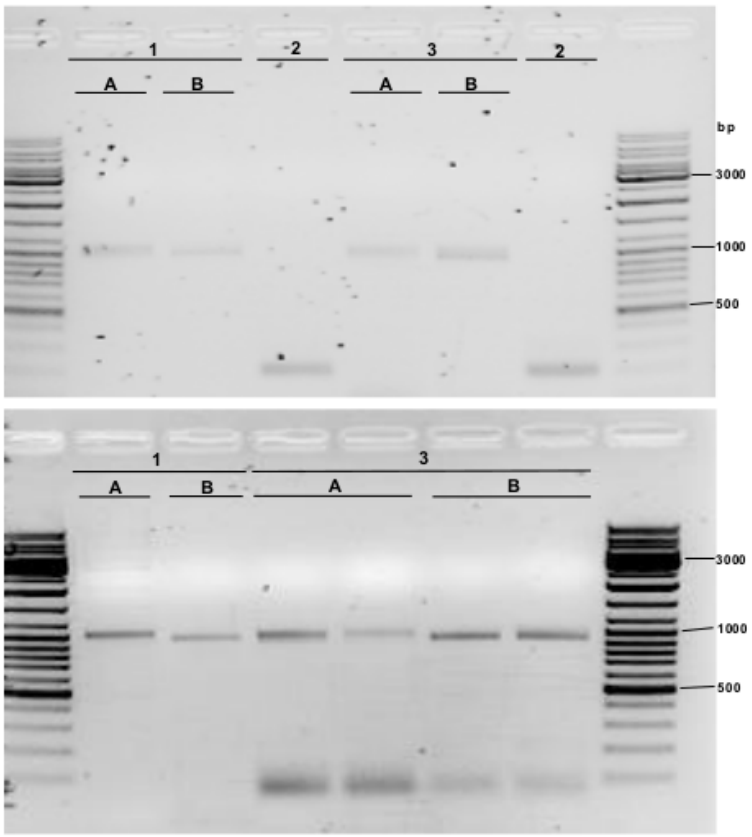


Figure 10: Amplification of the insert: 1) insert vector pIB; 2) UBC; 3) insert vector pMIB; A) PGIP 1; B) PGIP 2

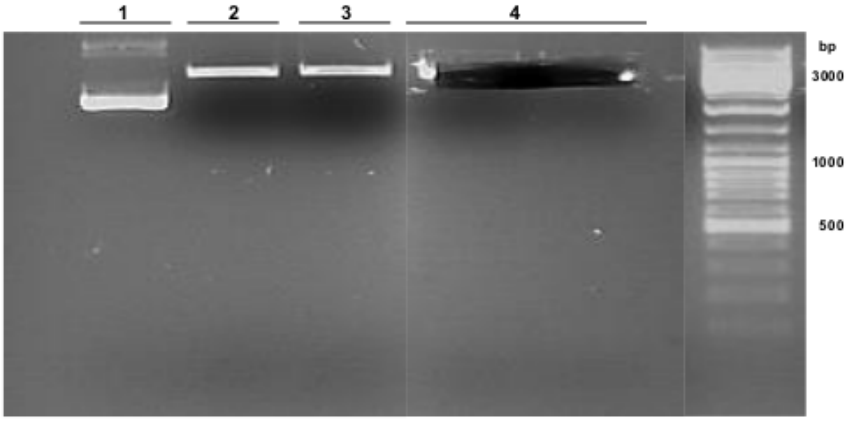


Figure 11: pMIB vector restriction: 1)uncut vector; 2) vector cut with Kpn I; 3) vector cut with Not I; 4)vector cut with Kpn I and Not I (picture after cutting)

5.4 Colony PCR and Sequencing

For preselection of candidate colonies that were constructed before (section 5.3) before the sequencing step, eight colonies were tested per vector and PGIP. The intention was to preselect four colonies per vector and PGIP, for the pIB vector, five PGIP 1 plasmid containing colonies were present, only one colony with PGIP 2 plasmid. For the pMIB construct two PGIP 1 containing and 4 PGIP 2 plasmid containing colonies were identified [see Figure 12]. The colony PCR was repeated to get more candidate colonies for pIB PGIP 2 and pMIB PGIP 1.

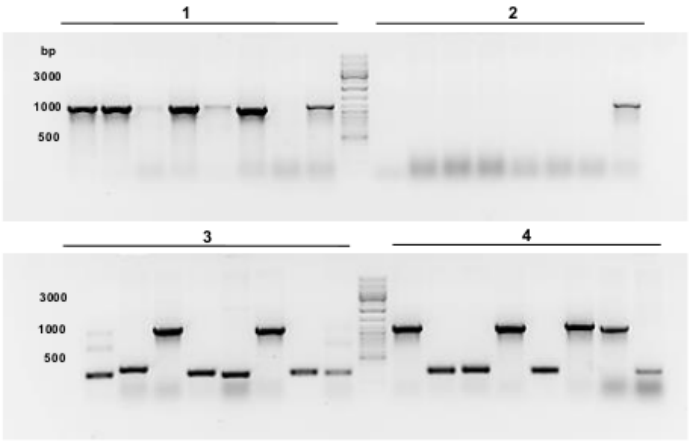


Figure 12: First colony PCR: 1) vector pIB PGIP 1; 2) vector pIB PGIP 2; 3) vector pMIB PGIP 1; 4) vector pMIB PGIP 2

After the second colony PCR four colonies for each construct could be provided, except for the pIB vector with PGIP 1 plasmid, for which only three colonies could be identified [see Figure 13].

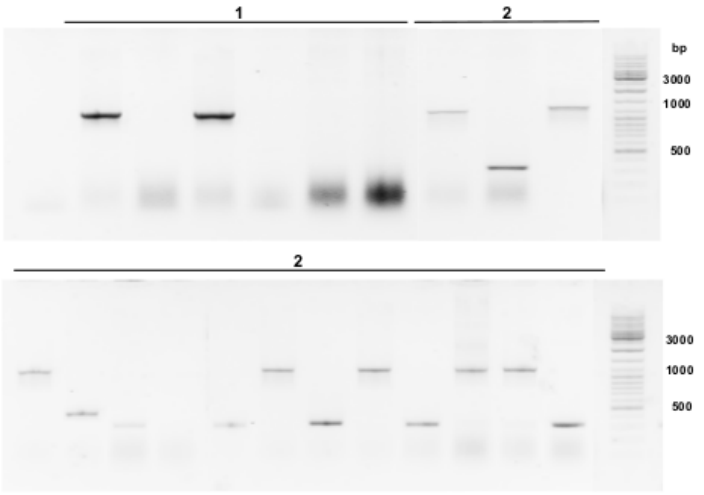


Figure 13: Second colony PCR: 1) vector pIB PGIP 2; 2) vector pMIB PGIP 1

. In the next step, the plasmid of selected candidate colonies were sequenced.
The comparison of the sequence of the proteins and the sequence of the plasmids from the transformed colonies showed that for PGIP 1 there is one candidate plasmid per vector construct and for PGIP 2 three candidate plasmids per vector were available. These plasmids were used for the transfecion of the Sf9 cells.

5.5 Western Blot

The, in section 5.4 selected, AtPGIP plasmids were used for a transfection of insect cells to express the PGIPs. To verify the expression of the PGIPs the SDS-Page and a following Western Blot was done. For each sample a fraction before binding to the V5-beads, an unbound fraction, a washing fraction and the bound V5 bead fraction was applied on the SDS gel. With these samples a successful expression and the binding to the V5-beads can be proved at once. The successfully V5-bound AtPGIP samples could later be used to bind them on a column for interaction studies. The positive control was a glycoside hydrolase of the family 45. The film was exposed to the samples for one hour and 15 min.

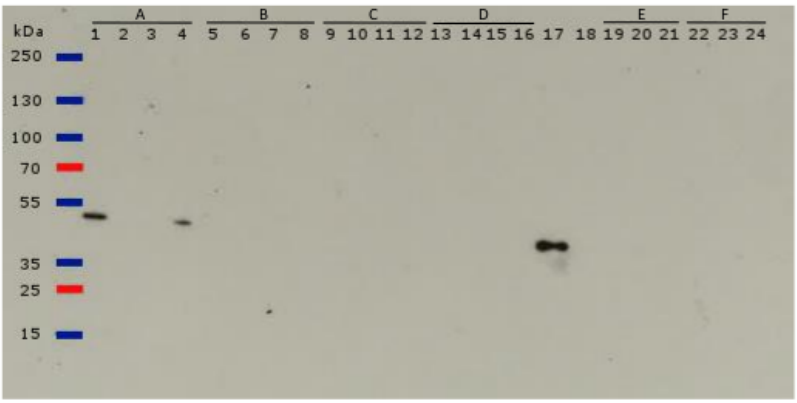


Figure 14: First Western Blot: order for every sample: before binding, unbound, washing fraction, bound to V5; A) pIB PGIP 1; B) pIB PGIP 2 pool of all three samples; C) pMIB PGIP 1; D) pMIB PGIP 2 pool of all three samples; 17) positive control; 18) negative control; E) pIB PGIP 2 three separate samples, before binding; F) pMIB PGIP 2 three separate samples, before binding

A band for the PGIP 1 unbound and V5 bound fraction and the positive control were detected. In accordance with UniProt, the size of about 40 kDa for the PGIP 1 and 36 kDa for the positive control were confirmed [see Figure 14]. To maximise the expression a retransformation of *E.coli* with both PGIPs of the pIB vector with a following Midi prep and a 6-Well transfection was performed. In addition an already existing plasmid of PGIP 3 of *Brassica napus* was also amplified for the SDS Page and the Western Blot. The gel was loaded with the fractions as described above. After one hour following bands could be seen [Figure 15].

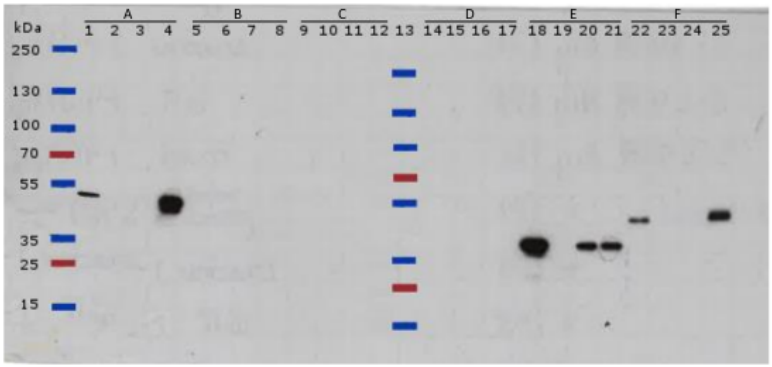


Figure 15: Western Blot after retransformation: order for every sample: before binding, unbound, washing fraction, bound to V5; A) pIB PGIP 1; B) pIB PGIP 2-1; C) pIB PGIP 2-2; D) pIB PGIP 2-3; E) positve control; F) PGIP 3 *Brassica napus*

The expression was successful for the PGIP 1 of the pIB vector in the unbound and V5-bead bound fraction, the positve control in the unbound, washing and V5-bead bound fraction and the PGIP 3 from *Brassica napus* in the unbound and V5-bead bound fraction. The sizes on the film corresponded to the sizes of the proteins with about 40 kDa for PGIP 1, 36 kDa for the positive control and 40 kDa for the PGIP 3 from *Brassica napus*.

6 Discussion

The PG and PGIP interaction plays an important role in plant defence. Pathogens rely on PG activity to invade the plant and to liberate nutrients at the same time. In this context little is known about phytophages and their PGs. To unravel the exact mechanism of the general interaction of PGs and PGIPs, a high resolution 3-D structure of the PG-PGIP complex is needed, which is not available at this time [31]. Nevertheless it is known, that PGIPs inhibit PGs with different mechanisms and that the residues of PGIPs play an important role for building the complex [5], [31]. While the research was mainly focused on the PG-PGIP interaction between plant PGIPs and microbial PGs, more and more studies involving insect PGs are published.

The inhibition effect towards microbial PGs is shown by plants overexpresssing PGIP genes, for example model plant *Arabidopsis thaliana* overexpressing both of their AtPGIPs and tobacco plants expressing the *Vitis vinifera* PGIP 1 showed significantly reduced symptoms caused by infection with *Botrytis cinerea* [25], [30] whereas the silencing of an AtPGIP caused enhanced susceptibility [25]. The inhibition of PGIPs of *Phaseolus vulgaris*, *Arabidopsis thaliana* and *Glycine max* in combination with the PGs of mirid bugs was tested 2006, resulting in the fact, that two of the *Phaseolus vulgaris* PGIPs inhibit all of the tested mirid bugs PGs [26]. The two AtPGIPs were also tested with the mirid bug PGs, but no inhibition could be detected. Nevertheless it is important to test the interaction and inhibition of AtPGIPs and PGs of *Phaedon cochleariae* because of their varying location and use: the mirid bugs may use the PGs for pre-oviposition stylet probing, to soften the plant material before ovipostion, whereas the PGs of *P. cochleariae* are detected in the gut which suggests a digestive function.

The result of the performed feeding assay on hole plants show that the beetles feeding on WT plants gained more weight than the beetles feeding on the PGIP knock out mutants. This fact could lead to the hypothesis that the PGIPs have a direct or indirect negative influence on the beetles weight gain for example by inhibiting the beetles PGs or influence the plant pathways. The hatching time was documented, but there was no significant difference between the beetles feeding on the three lines. A lengthen of the instar and a decrease of pupate state is already described for lepidopteran larvae, whereas the focus of this study was examining the influence of glucosinolates and their toxic breakdown products on the larvae of two generalist-feeding caterpillars [29]. Related to this glucosinolate study, another one deals with the *A. thaliana* glucosinolates in combination with *P. cochleariae* feeding and performance. The results show, that the

performance of *P. cochleariae* on mutants lines containing only aliphatic glucosinolates was worse and that the feeding of the beetles induced aliphatic glucosinolates in the tested mutant lines [38].
After hatching the beetles of the feeding assay were collected and fed with *Brassica rapa subsp. pekinensis* until they laid eggs. The number of eggs and also the number of second generation larvae was not influenced by feeding on the different *Arabidopsis* lines. The cause of this observation may be due the fact, that adult *P.cochleariae* are able to compensate a poor larval nutrition within 10 days and that the larval host plant plays no significant role in comparison to the adult host plant [39] or that the lab population is raised under optimal conditions . In nature, the beetles compete with other herbivores for their food, which can influence the performance.
To investigate if the differential weight gain is influenced or compensated by the amount of fed plant material, plant disc assays were performed. The amount of fed leaf material was expressed as leaf area. This would have been not possible by feeding on the whole plant. The beetles of this 18 h plant disc assay fed significantly less on the PGIP 2 mutant line, compared to the WT and the PGIP 1 mutant line. In combination with the feeding assay results, the beetles feeding on the PGIP 1 mutant would feed more leaf material to compensate the negative effect of the PGIP 2 as they gained the same weight like the PGIP 2 mutant feeders. This could lead to the hypothesis that AtPGIP 2 has more influence on the performance of the beetles. Unfortunately, a direct comparison with the feeding assay on hole plants is not possible, because of the different setups and individuals of the assays. Anyway the results gave an impulse to set up an additional plant disc assay, in which both, weight gain and fed leaf area are documented to underline the trend seen in the earlier assays. This assay was set up, starting with neonates of *P.cochleariae* and ending with the adults to see if there may be an accumulative effect on the metabolism over the larval instars. Previous studies have already shown the influence of the age of the diet on the gained body mass of *P.cochleariae* resulting in a higher body mass and a shorter development time of individuals feeding on young cabbage leaves [40]. In addition to that compensatory feeding behaviour of *P.cochleariae* was observed for larvae feeding on diet with lower food quality [54]. The results of the second plant disc assay (from neonate to adult), however, show that larvae feeding on WT, PGIP 1 mutant and PGIP 2 mutant leaf discs until the third instar, have no significant difference in weight, fed leaf area. The quotient of the weight and the fed area represents the gained body mass per leaf area as indicator for the metabolic rate, not just the gained weight like in the hole plant feeding assay. The results of the quotient

of the mutant feeder and the WT feeders are showing no significant difference. Neither compensatory feeding nor another influence of the three *Arabidopsis thaliana* lines was noticed. Following from these results the AtPGIPs would have no influence on the body mass. To underline the first hypothesis the small number of replicates that survived through the plant disc assay from neonate to adult should be mentioned. An assay with a higher number of replicates could differ in the results.

Reasons for the high mortality rate of the second plant disc assay could be the lack of moisture given by the plant discs or the influence of secondary metabolites of the plants. The family of Brassicaceae possesses glucosinolates as defend compounds, *Arabidopsis thaliana* contains more than 30 different glucosinolates, differently represented in the stages of development [12]. The glucosinolate itself is not toxic, but hydrolysation by the enzyme myrosinase, also produced by the plant, can result in toxic products. Induction of the glucosinolates comes along with plant damage, which was done in the plant disc assays by cutting the plant discs. Furthermore, in the roots of *Arabidopsis*, a connection of PGIP 1 and the indole-glucosinolate biosynthesis pathways is observed [48], even if there is no difference in the results between the PGIP 1 expressing WT and the PGIP 2 mutant and the PGIP 1 mutant, not expressing PGIP 1. Although specialists are able to detoxify the glucosinolates for example by prevention of the hydrolysis or the prevention of the formation of toxic products, even if the exact process of detoxification by *P.cochleariae* is unknown [40]. For lepidopteran larvae the detoxification increases with the age, transferring these observations to *P.cochleariae* larvae, this, in combination with the lack of moisture, could be an explanation for the early death of the neonates, even if the glucosinolates had no influence on lepidopteran larval survival. In the same connection a lengthen of instars and a decreased pupate state could be noticed for the lepidopteran larvae [29]. This results could neither be proved by the hatching time of the feeding assay, nor the time of the instars of the second plant disc assay.

PGIP expression is already performed for various PGIPs, in vitro in procaryotic systems as well as in vivo in different plant systems (see table 1). To investigate the expression of AtPGIPs, they were attempted to be expressed with insect (Sf-9) cells. Two different vectors were used and the result of the Western Blot shows, that the expression of AtPGIP 1 was successful with the pIB vector, which makes it the first PGIP expressed in cell culture. The expression with the pMIB vector was neither successful for both AtPGIPs. This result could be explained by the origin of the signal peptides used for the expression. The pMIB signal peptide was already included in the vector, in combination with the pIB vector, the native signal peptide of the PGIPs were used.

Following studies, like binding assays could be performed with these expressed AtPGIP 1 to investigate the PGIP-PG interaction with *Arabidopsis thaliana* PGIPs and PGs from *Phaedon cochleariae*.

7 Conclusion and outlook

In conclusion, PG-PGIP interaction plays a crucial role in plant herbivore interactions. An influence of *A. thaliana* PGIPs on the performance and feeding behaviour of *P. cochleariae* could be noticed by the performed feeding assay and the 18 h plant disc assay. The results of the feeding assay from neonate to adult could not underline this hypothesis, but it should be considered, that another assay like this with a higher number of replicates may differ in results. Next steps following the performed studies could therefore be plant disc assays with optimised conditions.
Another propose of this study was to express the AtPGIPs in insect cells and bind them to V5-beads. To get the optimal expression two vectors with different signal peptides were used. The expression and binding to the V5 beads of AtPGIP 1 was successful with the native signal peptide in the pIB vector, whereas no expression could be detected for the pMIB vector with included honeybee melittin secretion signal. The expressed AtPGIP 1 bound to the V5-beads could be used to perform binding assays with PGs or even closer to the insect system, with larval gut content, to improve knowledge of PG-PGIP interaction.
By further research, improvements in fight against plant pests, for example by genetic modified plants, could be archived. But not only in plant breeding this research could find application: PGs are commonly used in industrial processes in food industry, such as juice clarification, as enzyme in mash treatment while brewing, in distillery processing, bakery or even in sewage treatment. Plant PGs and PGIPs are also involved in fruit ripening.[34] ,[50], [7]) In all of these sections the PG or PGIP performance could be influenced by enhanced knowledge of interaction.

8 Acknowledgements

I want to thank the hole ENT group for the nice work atmosphere, especially Dr. Yannick Pauchet and Bianca Wurlitzer for their advice and help. Thanks to the greenhouse team of the Max-Planck-Institute for Chemical Ecology for their help with the plant rearing. Many thanks go to my advisor, Dr. Roy Kirsch, for his excellent planning, organisation, recommendations and help with my bachelor thesis. I also want to express my gratitude to Tim Krampitz and my hole family for their support.

9 Declaration

I hereby testify that the bachelor thesis with the topic "Studying the Influence of *Arabidopsis thaliana* PGIPs on Performance of *Phaedon cochleariae*" is written independently by my self. All passages taken from a source, whether verbatim or in substance, have been indicated as such. This thesis was not previously presented to another examination board and has not been published.

10 Tables and figures

		product size (in bp)
PCR 1	gene specific f & r PGIP 1	1140
	gene specific f & r PGIP 2	1025
PCR 2	T-DNA specific f, gene specific r PGIP 1	525
	T-DNA specific f, gene specific r PGIP 2	480

Table 2: Primer combinations for genotyping of *Arabidopsis thaliana*

Quick load *Taq* 2x Master Mix	12.5	µl
10 µM forward primer	0.5	µl
10 µM reverse primer	0.5	µl
template	1	µl (≃10 ng)
Nuclease-free water	10.5	µl
$\sum$	25	µl

Table 3: Set up for genotyping PCR

temperature	time
95 °C	0:30 min
95 °C	0:20 min
50 °C	0:30 min
68 °C	1:20 min
68 °C	5:00 min
4 °C	∞

Table 4: Genotyping of *Arabidopsis thaliana*: Program for thermocycler

Quick load *Taq* 2x Master Mix	12.5	µl
10 µM forward primer	1.25	µl
10 µM reverse primer	1.25	µl
template	1	µl
Nuclease-free water	9	µl
$\sum$	25	µl

Table 5: Set up for prooving PCR

98 °C	0:30 min
98 °C	0:10 min
53 °C	0:20 min
72 °C	0:40 min
72 °C	2:00 min
4 °C	∞

Table 6: Genotyping of *Arabidopsis thaliana* prooving PCR: Program for thermocycler

	before optimizing		after optimizing	
template	1	µl	2	µl
Primer Mix	1	µl	1	µl
Accu Prime Taq DNA Polymerase	0.2	µl	0.2	µl
buffer	5	µl	5	µl
water	42.8	µl	41.8	µl
	50	µl	50	µl

Table 7: conditions for the pIB vector before and after optimizing

Before optimizing (30 cycles)	After optimizing (35 cycles)	time
94 °C	94 °C	0:30 min
94 °C	94 °C	0:20 min
53 °C	53 °C	0:20 min
68 °C	68 °C	1:20 min
4 °C	4 °C	∞

Table 8: program for thermocycler: insert for pIB vector

template	2	µl
Primer Mix	2.5	µl
Master Mix Q5 High Fidelity	25	µl
water	20.5	µl
$\sum$	50	µl

Table 9: conditions for the pMIB vector

temperature	time
98 ℃	0:30 min
98 ℃	0:10 min
53 ℃	0:20 min
72 ℃	0:40 min
72 ℃	2:00 min
4 ℃	∞

Table 10: program for thermocycler: insert for pMIB vector

	PGIP 1	µl	PGIP 2	
insert	21.6	µl	21	µl
Not I	2	µl	2	µl
Kpn I	2	µl	2	µl
buffer	5	µl	5	µl
water	19.4	µl	20	µl
∑	50	µl	50	µl

Table 11: Restriction digest pMIB insert

vector	4	µl (2µg)	1	µl (500ng)	1	µl (500ng)
Not I	2	µl	1	µl	-	µl
Kpn I	2	µl	-	µl	1	µl
buffer	5	µl	5	µl	5	µl
water	37	µl	43	µl	43	µl
∑	50	µl	50	µl	50	µl

Table 12: Restriction digest pMIB vector

	PGIP 1		PGIP 2	
insert	0.31	µl	0.2	µl
vector	0.2	µl	0.2	µl
salt solution	0.5	µl	0.5	µl
water	1.99	µl	2.1	µl
∑	3	µl	3	µl

Table 13: Insertation setup for pIB vector construct

insert	2	µl
vector	2	µl
ligase	1	µl
buffer	2	µl
water	13	µl
$\sum$	20	µl

Table 14: Ligation for pMIB vector

Quick load *Taq* 2x Master Mix	12.5	µl
primer mix	0.5	µl
water	12	µl
$\sum$	25	µl

Table 15: Conditions for colony PCR

temperature	time
95 °C	2:00 min
95 °C	0:20 min
52 °C	0:30 min
68 °C	1:20 min
4 °C	∞

Table 16: Program for thermocycler: colony PCR

	24-well plate		6-well plate	
Plasmid	0.6	µg	2.4	µg
FuGENE® Transfection Reagent	1.8	µl	7.2	µl
medium	Add to volume of 25 µl		Add to volume of 100 µl	

Table 17: Setup for transfection with FuGENE®

4x sample buffer	2.5	µl
20x reducing agent	0.5	µl
10 % SDS	1	µl
sample	6	µl
$\sum$	10	µl

Table 18: SDS-PAGE sample preparation

S1			S2		
100mM Tris pH 8.5	5	ml	100mM Tris pH 8.5	5	ml
hydrogen peroxide	3	µl	luminol	50	µl
			coumaric acid	22	µl

Table 19: ECL solution (mixed directly before use)

11 Acronyms

AtPGIP	*Arabidopsis thalilana* polygalacturonase-inhibitor protein
bp	basepairs
cDNA	complementary DNA
cm	centimetre
Da	Dalton
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
gDNA	genomic DNA
GH	glycosyl hydrolase family
HCl	hydrochloric acid
Hz	Hertz
h	hour
min	minute
ml	millilitre
µl	microlitre
m	mutant
M	molarity
NaCl	sodium chloride
OG	oligogalacturonide
PCR	polymerase chain reaction
PGIP	polygalacturonase-inhibitor protein
PG	polygalacturonase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
tDNA	transfer DNA
UBC	ubiquitin C
V	Volt
WT	wild type
xg	gravitational force

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