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The Role of PGIPs in Plant Defense: Gene Regulation in Response to Biotic/Abiotic Stress and Binding to Mustard Leaf Beetle PGs

Bachelorarbeit

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3. ABBREVIATIONS

A. thaliana	*Arabidopsis thaliana*
AtPGIP	PGIP of *Arabidopsis thaliana*
BnPGIP	PGIP of *Brassica napus*
bp	base pair
BvPGIP	PGIP of *Beta vulgaris*
CAZy	Carbohydrate-Active EnZymes
cDNA	complementary DNA
CWDE	plant cell wall degrading enzymes
Da	Dalton
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
e.g.	for example
ECL	Enhanced chemiluminescence
ef1α	Elongation factor 1-α
eGFP	Enhanced green fluorescent protein
FWD	Forward primer
gDNA	genomic DNA
GH-28	Polygalacturonase belonging to the family 28 of glycoside hydrolases
GOI	Gene of interest
H_2O	Nuclease-free water
H_2O_2	Hydrogen peroxide
HG	Homogalacturonan
MAMP	Pathogen-associated molecular pattern
OG	Oligogalacturonide
P. cochleariae	*Phaedon Cochleariae*
PAMP	Pathogen-associated molecular pattern
PCO	*Phaedon cochleariae*
PCOGH28	Polygalacturonase belonging to the family 28 of glycoside hydrolases secreted by *Phaedon cochleariae*
PCR	Polymerase chain reaction
PG	Polygalacturonase

PGIP	Polygalacturonase-inhibiting protein
PIMS	Pectin integrity monitoring system
PVDF	polyvinylidene difluoride
PvPGIP	PGIP of *Phaseolus vulgaris*
REV	Reverse primer
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T-DNA	Transfer DNA
TAE-Buffer	Tris-Acetate-EDTA-Buffer
TBS-Buffer	Tris buffered saline
TG-Buffer	Tris-Glycine-Buffer
ubc	Ubiquitin C
v/v	Volume/Volume

4. INTRODUCTION

4.1 Plant defense against herbivorous insects and pathogens

Due to the food and energy production plants play an important role for all living organisms. Since about 350 million years, they have coexisted with herbivores and were confronted with biotic and abiotic stress from the environment [1]. According to the coevolution theory of Ehrlich and Raven, the interaction between plants and herbivores leads to chemical diversity in these species [2]. This interaction provides plants fertilization [3], whereas the goal for insects is to get food and oviposition[4]. However, not all herbivores are harmless to the host plant. Many of them try to damage it and even lead the plant to cell death. During the evolution, plants were also attacked by microbial pathogens and have established mechanisms to counteract against invading pathogens and herbivorous animals [5]. Despite the immobility and lacking immune system [6, 7], they were able to survive the battle with herbivores and pathogens. Otherwise it would have had dramatic effects on agricultural systems. Reason for this survival is evolving various plant defense mechanisms. These defense strategies can be categorized in constitutive and inducible defense [1]. Regardless of the attack of pathogens and herbivores plants produce constitutively physical armaments such as trichomes or thorns and synthesize chemical products including e.g. glucosinolates, terpenes and alkaloids [8]. These secondary metabolites can be toxic or antidigestive to pathogens and herbivores. In contrast, induced defense is activated by the presence of pathogens and herbivores [9]. In order to fend off biotic and abiotic stress, first plants have to recognize the attack.

4.2 Recognition strategies of microbial invasion

In order to activate defense responses for the survival of the plant, the presence of pathogens and herbivores has to be recognized immediately. Innate immunity allows plants to perceive pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by pattern recognition receptors (PRR) [10]. Such P-/MAMPs can be chitin from fungi or flagellin from bacteria [11]. This detection triggers an intracellular signaling cascade, which leads to the regulation of expression of defense related genes and the induction of defense responses, so called PAMP-triggered immunity (PTI) [12]. Plants are also capable of sensing pathogen and herbivore invasion by recognizing

damage-associated molecular patterns (DAMPs) caused by tissue damaging such as microbial attack and abiotic stresses. DAMPs can act as elicitor of defense responses inducing e.g. glucanase, chitinase as well polygalacturonase-inhibiting protein (PGIP) [13], reactive oxygen species or nitric oxide [11, 14]. Endogenous phytohormones including salicylic acid, jasmonic acid and ethylene are also induced [15]. These induced defense genes counteract the microbial attack.

4.3 Role of plant cell walls in plant-pathogen interaction

Plant cell walls play an important role in plant growth, cell differentiation, intercellular communication and defense against herbivores and pathogens [16]. It consists primarily of a structurally complex network of polysaccharides such as celluloses, hemicelluloses and pectins [16]. Pectin embeds the cellulose-hemicellulose network and is therefore the most accessible structure of the plant cell wall [17] used to attack by pathogens. This explains the consequently monitoring of the pectin status by the 'pectin integrity monitoring system' known as PIMS [13]. The main pectic polysaccharides include rhamnogalacturonan I, rhamnogalacturonan II and homogalacturonan (HG) known as the primary target [16, 18]. HG is a linear chain of (1-4)-linked α-D-galacturonic acid residues containing some methylesterified carboxyl groups [17, 19]. It plays an important role in affecting cell adhesion and tissue integrity [20].

The plant cell wall is an important interface between plant and microbes. As a first line of physical barrier, pathogens have to penetrate the plant cell wall in order to gain nutrients [20]. Due to the coevolution, plants have generated various defense strategies (4.2), whereas pathogens also evolved mechanisms to counteract the plant defense [17, 20]. In order to breach the cell wall, pathogens secrete plant cell-wall degrading enzymes (CWDE). Based on their expression and functional specialization, they are categorized in different families of glycoside hydrolases (GH), which can be found in the Carbohydrate-Active EnZymes (CAZy) database [21]. CWDEs are produced in insects, fungi, nematodes and phytopathogenic bacteria [22]. However, it has to be mentioned that until recently production of PGs by insects was rarely determined due to the fact that model genomes such as *Tribolium castaneum* and *Bombyx mori* doesn't encode CWDEs [23]. Nevertheless, discovery and sequencing of CWDEs secreted by insects increased in a while [23], which can be explained with horizontal gene transfer from microorganisms such as ascomycete fungi [24]. Among the CWDEs, pectin degrading enzymes are the

first enzymes secreted by pathogens in order to weaken and degrade the plant cell wall [17]. Best known pectin degrading enzymes are polygalacturonase, pectate lyases or pectin methylesterases [17].

4.4 Polygalacturonase as important virulence factor during pathogenesis

It is well-known that polygalacturonases (PG; E.C. 3.2.1.1.5) are secreted at early stages of the microbial attack [25], which explains the importance of studying these enzymes. Due to their sequences and structure [26] PGs belong to the GH28 family. They cleave the linkages between D-galacturonic acid residues in homogalacturonan [27], which leads to cell wall degradation and tissue maceration [28]. Many microorganisms such as bacteria and fungi, but also other species like insects and nematodes produce genes encoding PGs [29]. In order to enhance the likelihood of successful HG degradation, microorganisms have evolved PGs with different isoforms. Based on their cell wall degrading ability, they play an important role as virulence factor during pathogenesis confirmed by many studies previously. Due to secreted PGs virulence of several fungi such as *Sclerotinia sclerotiorum* [30] and *Alternaria citri* [31] increased. In contrast, deletion of a gene encoding PG showed reduced virulence on tomato and broad bean [32]. The significance of PG was also reported for several bacteria [26]. Furthermore, it is claimed that PGs by phytophagous insects are responsible for plant damage [29]. As a consequence of the homogalacturonan degradation, PGs release many fragments, the oligogalacturonides (OG), which as best studied DAMPs may elicit induced defense responses against the microbial attack (5.2) [11].

4.5 Polygalacturonase-inhibiting proteins

4.5.1 Genomic organization

As described above, plants response to biotic and abiotic stress stimuli by induced defense responses. In case of HG degradation by PGs, it is well-known that plants produce PG-inhibiting proteins known as PGIPs, which were first published over 50 years ago [33]. PGIPs are extracellular leucine-rich-repeat (eLRR) proteins present in the plant kingdom, both in monocots and dicots [34]. They counteract PG's activity as well as favors the accumulation of elicitor active OGs followed by an enhanced plant defense response [13]. The expression of PGIP caused by pectin degradation indicates that PGIPs are important player in PIMS (4.3) [13]. PGIPs can be found as single gene but also encoded by small

gene families with different PGIP isoforms, which possess different recognition specifity. The family can be composed of two genes (*A. thaliana*) or even sixteen genes (*B. rapa*) [25]. Such gene families can be found in all plant species such as pear, soybean, bean or grape [25]. Phylogenetic analysis indicated duplication of these genes, which could be derived from a common ancestor [26]. Usually these genes are intronless, whereby some possess small introns, e.g. Arabidopsis [25]. In general, PGIPs are expressed in all plant tissues. Several studies showed that the expression level depends on the localization. For example, BnPGIPs are both low expressed in leaves [35].

4.5.2 Gene regulation and role of PGIPs in plant defense

Since PGIPs are encoded by gene families with different isoforms they are also differentially regulated in response to biotic and abiotic stress stimuli such as feeding by herbivores or wounding. For example, while BnPGIP1 is upregulated in response to flea beetle, BnPGIP2 doesn't show any upregulation [35]. On the other hand, mechanical wounding increases the expression of both genes. The same pattern can be also found in Arabidopsis for AtPGIP1 and AtPGIP2 [14]. Interestingly, these two genes are regulated through different signal pathways. The expression of AtPGIP1 is mediated by OG, whereas AtPGIP2 is upregulated in response to jasmonic acid [14]. "Independent regulation of different *pgip* genes may enhance the likelihood of defense gene activation during pathogen infection" [17]. Furthermore, several over- and anti-sense-expressing studies demonstrated the important role of PGIP in plant defense against fungal and bacterial invasion: Overexpressing of Arabidopsis and bean PGIPs enhanced resistance to *B. cinerea* infection [14, 36]. In contrast, anti-sense expressing of PGIPs in Arabidopsis led to increased susceptibility against *B. cinerea* [37]. Overexpressing PGIPs from pear showed delayed development of Pierce's disease, caused by bacteria *Xylella fastidiosa* [38]. Additionally, the expression level of PGIPs can be considered as a correlation to susceptibility of plants to microorganisms. Increasing expression levels of PGIP correlates with enhanced resistance [39]. In general, many times it has been successfully shown that PGIPs may have big influence to plant defense.

4.5.3 Interaction of PGIP with PG

Previous studies confirmed inhibition of fungi and some insect's PG (which were tested with crude extracts and not pure proteins), but not the one secreted by plants or

bacteria [17]. In order to investigate the inhibition of PG by PGIP, PGIPs have to be successfully expressed first. Positive inhibitory activity against PGs secreted by fungi could have been proved more often than the inhibition of insects' PG. However, D'Ovidio et al. showed that in bean PvPGIP3 and PvPGIP4 can inhibit two mirid bugs, *Lygus rugulipennis* and *Adelphocoris lineolatus* [40]. This inhibition activity against PGs secreted by these two mirid bugs couldn't report PGIPs from Arabidopsis [41] and Soybean [42]. Furthermore, a citrus' PGIP was able to inhibit PG from *Diaprepes abbreviates* [43]. In some cases, PGIPs didn't show an inhibitory activity even if the expression was successfully [25]. Not only do PGIPs differ in their gene regulation, but also in the inhibitory activity. For example, while *B. cinerea* is inhibited by all four PGIPs in *P. vulgaris* (PvPGIP), PG from *A. niger* is only inhibited by PvPGIP1, 2 and 4 [17].

4.6 Study systems

4.6.1 *Arabidopsis thaliana*

As a member of the mustard family (Cruciferae or Brassicaceae), *Arabidopsis thaliana* is distributed throughout Europe, Asia, North America, East Africa, Australia and Japan [44]. In year 2000, the 115 Mbp genome was completely sequenced [45], which is organized in five chromosomes [46]. Kaul et al. also determined about 25.000 genes encoding proteins from 11.000 families [45]. Total development time from seed germination to maturation of the first seeds is completed in six weeks [46]. *A. thaliana* can be grown in petri plates or in pots, for both required location in a greenhouse or under fluorescence light [46]. Due to the complete genome sequencing *A. thaliana* has become an important model system for plant defense studies in plant-heterotrophs interactions. Ferrari et al. analyzed two PGIP genes in *A. thaliana*, AtPGIP1 and AtPGIP2. These two genes are tandemly located on Chromosome 5 with a short distance of 507 bp. Both of them possess a short intron [14]. In this study, *A. thaliana* was used to investigate the gene regulation in response to biotic and abiotic stress stimuli.

4.6.*2 Phaedon cochleariae*

Phaedon cochleariae, the mustard leaf beetle, belongs to the family of Chrysomelidae and is widespread in Western Europe, Anterior and Middle Asia, and North America. It is known, that they feed on various plants of the family *Brassicaceae* such as cabbage, horseradish and radish. Preferred ecological conditions are 22-25 C with a relative

humidity of 70-80 %. Life cycle lasts on average 33 – 37 days [47]. Kirsch et al. showed 9 transcripts encoding Polygalacturonase as putatively active cell-wall degrading enzyme secreted into the gut by feeding on plants [22]. The relatively short life cycle and the presence and secretion of Polygalacturonase make *P. cochleariae* a suitable system in study plant defense against herbivores.

5. AIMS OF THIS STUDY

The aim of this study is, to investigate and compare the PGIP gene regulation in response to biotic and abiotic stress using the plant model *Arabidopsis thaliana.* Biotic stress stimuli are conducted by putting *Phaedon Cochleariae* beetles and larvae on the plants, whereas abiotic stress should be performed by mechanically wounding the plants. In this study, three different plant lines should be tested for every treatment. One of these is ecotype Columbia-0 containing PGIP1 and PGIP2, the other two are mutant lines with knock out of one PGIP gene by transfer-DNA (T-DNA) insertion, respectively. Gene expression levels should be analyzed by extracting mRNA of each plant line and treatment, reverse transcribing them in cDNA, which are used as template for real time PCR.

Another goal of this study is, to investigate the interaction of AtPGIP1 with eight PGs secreted by *Phaedon Cochleariae* (PCOGH28). The interaction should be tested by expressing AtPGIP1 and PCOGH28 with different tags in Sf9cells followed by a binding assay. Results could be analyzed by western blotting, whereby previously bands have to be separated with SDS-PAGE.

6. MATERIALS AND METHODS

6.1 Materials

6.1.1 Devices

Name	**Producer**
2100 Bioanalyzer	Agilent Technologies, Waldbronn
Avanti ™ J-20 XP Centrifuge	Beckman Coulter GmbH, Krefeld
Centrifuge 5424 R/ 5415 R/ 5810 R	Eppendorf, Hamburg
Certomat IS	Sartorius AG, Göttingen
CFX Connect™ Real-Time PCR System	Bio-Rad Laboratories GmbH, München
Criterion™ Blotter 560BR	Bio-Rad Laboratories, Inc., USA
CRITERION™ CELL	Bio-Rad Laboratories, Inc., USA
Dark Reader® DR196 Transilluminator	Clare Chemical Research Inc., USA
Digital Heat Block	VWR International, USA
EB2E climate chamber	Snijders scientific, The Netherlands
EPgradient S Thermocycler	Eppendorf, Hamburg
GeneGenius 2 Gel Imaging System	Syngene International Ltd., India
Mastercycler epgradient S	Eppendorf, Hamburg
MC1000 HE-EVD climate chamber	Snijders scientific, The Netherlands
Nanophotometer N60	Implen GmbH, München
P20 Minicell Power Pack	Biometra GmbH, Göttingen
QikSpin Mikrocentrifuge	Süd-Laborbedarf GmbH, München
Stuart Rotator SB3	Cole Palmer, UK
Subcell GT (wide mini)	Bio-Rad Laboratories GmbH, München
Thermomixer Comfort	Eppendorf, Hamburg
TissueLyser LT	QIAGEN, Hilden
Vortex Genie 2	Scientific Industries, NY, USA
WaterBATH E5 5I	Dinkelberg Anayltics GmbH, Gablingen

6.1.2 Chemicals

Name	**Producer**
2-Propanol, Rothipuran ≥ 98 %	Carl Roth GmbH + Co. KG, Karlsruhe
6X Orange Loading Dye, 1 ml	Fermentas, USA
ABsolute Blue qPCR SYBR Green Mix Plus ROX Vial, 1,25 ml	Thermo Scientific, USA

Ampicillin sodium salt, Cellpure® ≥ 91 %	Carl Roth GmbH + Co. KG, Karlsruhe
Anti-GFP antibody (ab6673)	Abcam, UK
Carestream® Kodak® autoradiography GBX developer/replenisher	Sigma-Aldrich Chemie GmbH, München
Carestream® Kodak® autoradiography GBX fixer/replenisher	Sigma-Aldrich Chemie GmbH, München
EDTA 0.5 M, pH 8.0	Invitrogen™, Thermo Fisher Scientific GmbH, Bonn
Ethanol, Rothipuran ≥ 98 %	Carl Roth GmbH + Co. KG, Karlsruhe
Ethidium bromide 1 % (10 mg/ml)	Carl Roth GmbH + Co. KG, Karlsruhe
FuGENE® Transfection Reagent	Promega GmbH, München
Gentamycin (50 mg/ml)	AMRESCO LCC, USA
Goat anti-V5 Agarose Immobilized 0.1 mg antibody	Bethyl Laboratories, Inc., UK
Goat Anti-V5 agarose immobolized	Biomol GmbH, Hamburg
hydrochlor acid 37 %	Carl Roth GmbH + Co. KG, Karlsruhe
LiChrosolv® Water for chromatograhy	Merck KGaA, Darmstadt
Luminol for chemiluminescence	Sigma-Aldrich Chemie GmbH, München
Methanol, Rotipuran ≥ 99,9 %	Carl Roth GmbH + Co. KG, Karlsruhe
$NotI_{HF}$ (R3189S)	New England Biolabs Inc., Ipswich, Massachusetts
Nuclease free water, 50 ml	Ambion, USA
o'Gene Ruler DNA Ladder Mix, 0.1 µg/µl	Thermo Scientific, USA
p-Coumaric acid	Sigma-Aldrich Chemie GmbH, München
PageRuler™ Plus Prestained Protein Ladder	Thermo Scientific, USA
PERDROGEN® 30 % b / weight	Riedel-de, Haen GmbH, Seelze
Powdered milk, Blotting-grade, low-fat	Carl Roth GmbH + Co. KG, Karlsruhe
Q5® High Fidelity 2x MasterMix	New England Biolabs Inc., Ipswich, Massachusetts
Quick Load Taq 2x Master Mix	Biolabs, UK
Rothiphorese® 50x TAE Buffer	Carl Roth GmbH + Co. KG, Karlsruhe
SacII (R01757S)	New England Biolabs Inc., Ipswich, Massachusetts
SDS ≥ 99.5 % Blotting Grade	Carl Roth GmbH + Co. KG, Karlsruhe
SeaKem® LE Agarose	Lonza, USA
Sf-900™ III SFM	Gibco™, Thermo Fisher Scientific GmbH, Bonn
Sf9 cells in Sf-900™ III SFM	Gibco™, Thermo Fisher Scientific GmbH, Bonn

SOC Media	Amresco Inc., USA
T4 DNA-Ligase	New England Biolabs Inc., Ipswich, Massachusetts
Tris base ≥ 99.9 %	Sigma-Aldrich Chemie GmbH, München
Tris/Glycine Buffer, 10x	Bio-Rad Laboratories GmbH, München
TRIzol® Reagent, 100 ml	Ambion, USA
TURBO™ DNase 2 U/µl	Invitrogen, Lithuania
TURBO™ DNase Buffer 10X 1.75 ml	Invitrogen, Lithuania
V5 Tag Antibody (E10/V4RR), 50 µl (1 mg/ml)	Invitrogen™, Thermo Fisher Scientific GmbH, Bonn
XT MOPS, running buffer, 20x	Bio-Rad Laboratories, Inc., USA
XT Reducing Agent, 20x, 1 ml	Bio-Rad Laboratories, Inc., USA
XT Sample Buffer, 4x, 10 ml	Bio-Rad Laboratories, Inc., USA

6.1.3 Consumables

Name	**Producer**
6-/24 Well CytoOne® plate, untreated	STARLAB GmbH, Hamburg
96-Well PCR Plate, Non-Skirted	STARLAB International GmbH, Hamburg
Amersham Hyperfilm™ ECL	GE Healthcare Europe GmbH, Freiburg
Centrifuge Tubes with screw caps, 50 ml	Labcon, Hannover
ColiRollers™ Plating Beads	EMD Millipore Corp., USA
Eppendorf Conical Tubes 50 ml	Eppendorf, Hamburg
Hard-Shell® PCR Plates 96-well, thin-wall	Bio-Rad Laboratories, Inc., USA
PCR 8er-Softstrips 0.2 ml, farblos	Biozym Scientific GmbH, Oldendorf
Reaction Tubes 5 ml/ 2 ml/ 1.5 ml/ 0.5 ml	Eppendorf, Hamburg
Safe-Lock Tubes 1.5ml/0.5ml	Eppendorf, Hamburg
Stahlkugeln 3/32”	ASK Kugellagerfabrik Artur Seyfert GmbH, Korntal-Münchingen
SuperClear® tubes, 50 ml	Labcon, Hannover

6.1.4 Commercial *Kit*s

Name	**Producer**
DNA Clean & Concentrator™-5	Zymo Research, USA
GeneJET Plasmid Miniprep Kit	Thermo Scientific, Lithuania

InnuPREP RNA Mini Kit	Analytik Jena AG, Jena
mRNA Isolation Kit	Roche Diagnostics GmbH, Mannheim
PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time)	TaKaRa Bio Inc., Japan
PureLink™ HiPure Plasmid Filter Midiprep Kit	Thermo Fisher Scientific, Lithuania
RNeasy MinElute Cleanup Kit	QIAGEN, Hilden
TOPO TA Cloning® Kit For Sequencing, pCR®4-TOPO® Vector	Invitrogen, Lithuania
Verso cDNA Synthesis Kit	Thermo Fisher Scientific, Lithuania
Zymoclean ™ Gel DNA Recovery Kit (uncapped columns)	Zymo Research, USA

6.1.5 Software

Name	**Producer**
2100 Expert	Agilent Technologies, Waldbronn
CFX Connect™ Real-Time PCR Detection System 3.1	Bio-Rad Laboratories, Inc., USA
SeqManPro 15	DNASTAR, Inc., USA
NanoPhotometer® N 60	Implen GmbH, München
Editseq 15	DNASTAR, Inc., USA
SigmaPlot 12.0	Systat Software Inc.,
Adobe Illustrator CS5	Adobe Systems
Microsoft Excel	Microsoft Corporation

6.2 Methods

6.2.1 Plants and insect cultivation

Arabidopsis thaliana wildtype Columbia-0 (wt) are kept in stock in the greenhouse. The mutants with T-DNA insertion, GK_092G09.15 (PGIP1-mutant) and GK_717A02.02 (PGIP2-mutant), were ordered from the Nottingham *Arabidopsis* stock center (NASC). After seeding and infecting all of the plant lines with nematodes (*Steinernema feltiae)* the plants were stored for four days at 4 °C and were cultivated six weeks in a climatic chamber with a 10 h light/14 h dark photoperiod and relative humidity of 50 % at 21 °C. Plastic cups were put on the two weeks old plants to avoid any mechanical wounding during the growth and afterwards. Six weeks-old plants were used for all the experiments.

Larvae and adults of *Phaedon cochleariae* are kept as a sustained culture on leaves of Chinese cabbage. They are stored at 21 °C on a cycle of 16 h light/ 8 h dark.

6.2.2 Plant-Treatment-Assay

The wildtype (wt) and two mutant lines (PGIP1-mutant and PGIP2-mutant) were treated in three different ways under constant conditions for 20 hours at daylight. Six plants of each line were used for every treatment. Wounding experiments were done with sterile forceps on sixteen leaves per plant at the beginning, after 4 hours and after 8 hours. For feeding experiments, a mixture of *Phaedon cochleariae* larvae and adults were used. On average 10 larvae and 5 adults were placed on each plant. Non-treated plants represented control plants with steady state level of PGIPs. All of the plants were covered with curtains to avoid any other biotic or abiotic stress during the assay. After 20 h, leaves were harvested, immediately immersed in liquid nitrogen and stored at -80 °C. For RNA-Extraction three biological replicates of every treatment and line were collected in 1.5 ml safe-lock reaction tubes, so that in sum 27 replicates were ready to use for the extraction.

6.2.3 Genotyping of used plant lines

For Genotyping, at first the genomic DNA (gDNA) from the collected plant material of each line had to be extracted. Five biological replicates of each of the three lines were tested for the extraction. The isolation of the genomic DNA of the 15 samples was conducted according to Edwards' et al. protocol with slightly modifications [48]. Here, in this case the leaf disc sample in a 1.5 ml reaction tube containing three metal beads

was grinded in the TissueLyser LT at 50 Hz for 1 minute and afterwards centrifuged for 1 minute at full speed. All the other centrifugation steps were also carried out at full speed. At the end the pellet was dissolved in 100 µl nuclease-free water by gentle shaking in the Thermomixer at 300 rpm at room temperature. Concentration of the isolated gDNA was determined photospectrometically by using Nanophotometer N60. In order to verify the homozygous plant lines a PCR was realized with Quickload TAQ 2x MasterMix according to the user guideline [49]. Four different primer combinations were tested for the genotyping of the plant lines. The first two are specific to the PGIP1 and PGIP2 coding sequence, which can determine wildtype or heterozygous plant lines (Table 6). The other one can only amplify heterozygous or homozygous plant lines due to the fact that the forward primer only binds on the T-DNA, whereas the reverse primer is specific to the PGIP1 or PGIP2 gene (Table 7). Finally, five biological replicates for each of the three lines with four primer combinations were tested, so that in sum 60 replicates had to be prepared for PCR. All PCR setups have been performed using the same conditions (Table 5).

The PCR reactions were analyzed on a 1.2 % agarose gel and visualized by ethidium bromide staining. 400 ml TAE-agarose gel with 24 µl ethidium bromide was prepared. 5 µl per sample were loaded on the gel. 4 µl O'gene ruler DNA Ladder Mix was applied as DNA marker. After running 40 min at 160 V, the results have been analyzed with a gel imaging system.

6.2.4 RNA Extraction

Extraction with Trizol

Total RNA was extracted using TRIzol reagent according to the process outlined by the manufacturer [50]. Prepared RNA was dissolved in 89 µl nuclease free water. In order to eliminate any contaminating DNA a DNase treatment was performed with 1 µl Turbo DNase and 10 µl Turbo DNase Buffer. The treatment was carried out 30 minutes at 37 °C. RNA Purification was realized using RNeasy MinElute Cleanup Kit according to manufacturer's protocol started with 100 µl sample volume [51]. Afterwards RNA concentration was measured photospectrometically with Nanophotometer N 60. The presence of degrading products and the integrity of RNA was tested with Agilent RNA 6000 Nano Kit Guide [52]. A maximum of 200 ng of RNA was used for the quality check.

Extraction with InnuPrep RNA Mini Kit

Isolation of total RNA was performed using innuPREP RNA Mini Kit 2.0 slightly modified [53]. The method of liquid nitrogen was chosen for the homogenization and lysis. The contaminated DNA digestion was realized with 60 µl total RNA, 2 µl Turbo DNase, 10 µl Turbo DNase Buffer and 28 µl Nuclease free water for 1 h at 37 °C. Purification, concentration determination and the quality check of the total RNA samples were realized as described above (Extraction with Trizol).

Extraction with mRNA-Isolation-Kit

mRNA of the samples was extracted using mRNA Isolation Kit Version 08 with some changes [54]. 50 – 100 mg tissue was grinded in a precooled mortar and added to 1.2 ml chilled Lysis Buffer. In this experiment, 75 µl Streptavidin-coated magnetic particles were applied. The magnetic particles were washed two times with 250 µl Wash Buffer and at the end with 250 µl nuclease-free water. After washing the Streptavidin-coated magnetic particles, the mRNA was eluted in 25 µl water. The amount of mRNA was determined photospectrometically with Nanophotometer N 60.

6.2.5 cDNA-Synthesis

Verso cDNA

500 ng of total RNA samples were reverse transcribed in a 20 µl reaction using Verso cDNA Synthesis Kit based on the manufacturer's instructions with some modifications [55]. The cDNA synthesis was performed with two steps including initial denaturation and synthesis. RNA samples together with the primer mix Random Hexamer : Oligodt (3:1, v/v) were filled up with nuclease free water to a volume of 12µl followed by a denaturation for 5 min at 70 °C. The cDNA synthesis was carried out by adding the missing components to the denaturized samples. The samples were synthesized in a cycler with following conditions: 60 min at 42 °C, 30 min at 50 °C, 2 min at 95 °C and holding at 4 °C. All cDNA reactions were filled up with nuclease-free water to a volume of 100 µl and stored at -20 °C.

Takara PrimeScript RT reagent with gDNA Eraser

cDNA Synthesis was carried out using 100 ng mRNA according to the manufacturer's guideline slightly modified [56]. The treatment of gDNA elimination was conducted

30 min at 42 °C. All samples were filled up with nuclease-free water to a volume of 100 µl and stored at -20 °C until further use.

6.2.6 Real-Time PCR

All Real-time PCRs were performed in optical Hard-Shell® PCR 96-well plates using CFX Connect Real Time PCR Detection System, ABsolute Blue QPCR Mix SYBR Green (Table 1) and prepared cDNA samples as template. Before the start of the PCR, all plates were covered with Microseal® 'B' seal to avoid any contamination of the samples. For real time PCR, two gene specific primer combinations were designed. All primers were designed with SeqManPro15. The PGIP1 gene specific primer combinations can produce 212 bp amplicons, while the PCR product with PGIP2 coding sequence primer is 224 bp in length (Table 9). Elongation factor 1α (ef1α; AT5G60390) and Ubiquitin C (ubc; AT5G25760) were used as reference genes. Primer efficiency for the real time PCR primer indicated above was determined by serial dilutions of a wildtype cDNA sample. Here, every column represents the serial dilution with 100 % as output sample (Table 10). The efficiency factor of each primer pair was determined using the slope of the standard curve for the efficiency calculator [57]. The cycler was programmed to 95°C for 15 min; 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s; 95°C for 10 s; followed by a melting curve program from 55°C to 95°C in increasing steps of 0.5°C. Three plates were prepared with the same layout and amount of cDNA as template. Each of the plates belonged to the respective treatment (wounding, feeding and control) and filled up with three biological replicates of each of the three plant lines. All PCR reactions were performed in duplicate (Table 11). Here, the expression levels of PGIP1 and PGIP2 genes were determined by dissociation curve analysis. Quantities of the genes of interest (GOI) were expressed as RNA molecules of GOI/1000 RNA molecules of reference gene. Determined data were compared by one-way ANOVA and Tukey's honestly significant difference (HSD) test using SigmaPlot 12.0.

Table 1: Real-Time PCR setup

Component	***Volume***
SYBR Green Mix	12 µl
cDNA	1 µl
H_2O	9 ml
30 µM Primer Mix	3 µl
Σ	**25 µl**

Primerspecifity

Due to the fact that AtPGIP1 and AtPGIP2 are tandemly duplicated genes and not far apart from each other, it is necessary to verify the specifity of the primers designed for real time PCR. The cDNAs encoding the wildtype of the wounded plant line were amplified with the respectively primer combination (PGIP1- and PGIP2-specific). PGIP1 specific primer amplifies a product size of 212 bp and PGIP2 specific primer a product size of 224 bp (Table 9). Because of the small size difference, it is not possible to determine the primer specifity by loading the real-time PCR products on a gel and see the difference. Hence, the products had to be prepared for sequencing. 20 µl of each real-time PCR product was prepared with 4 µl Loading Dye. The real-time PCR reactions were analyzed on a 2 % agarose gel and visualized by ethidium bromide staining. 100 ml TAE-Buffer with 8 µl ethidium bromide was prepared. After running 30 min at 140 V a gel clean-up was realized according to the manufacturer's protocol (Gel DNA Recovery Kit, ZymoResearch, [58]). The purified PCR products were cloned in pCR™4-TOPO® using TOPO® TA Cloning® Kit for Sequencing slightly modified (Table 2). After plating the TOP 10 cells on LB-agar dishes containing 100 mg/ml Ampicillin over night at 37 °C some colonies were picked and added to 4 ml LB-Medium including also 100 mg/ml Ampicillin. The samples were shaked at 300 rpm at 37 °C overnight and were ready to use for the Mini-Preparation (Mini-Prep). The method of Mini-Prep was realized based on the manufacturer's protocol (GeneJET Plasmid Miniprep Kit, [59]) with some modifications. In this case, the precipitation of cell debris and chromosomal DNA was carried out 10 min at 16.000 g. The two washing steps were conducted at 16.000 g and the removal of the washing step at full speed. Positive clones were verified by Sanger sequencing.

Table 2: Setup for TOPO/TA-Cloning reaction

Reagent	***Volume***
Salt solution	0.5 µl
Gel extract	10 µl
pCR™4-TOPO®	1 µl
Nuclease-free water	add to 3 µl

Meanwhile, new primer combinations were designed including one exon-intron boarder spanning primer, which cannot amplify genomic DNA, but cDNA (Table 12). On account

of the fact that these primer combinations amplify product with differentiable size, it is sufficient to load the real-time PCR primer on a gel and analyze the primer specify. The method of gel-electrophoresis was carried out as described above.

Determination of genomic DNA amount in cDNA samples

In order to verify the expression of cDNA samples accurately, is has to be guaranteed that the amount of genomic DNA is as low as possible, so that it cannot influence the real time PCR results. For determination of genomic DNA amount in RNA samples, a real time PCR was performed using the same amount of RNA and cDNA as template and comparing the results with each other. RNA samples shouldn't get amplified unless the amount of genomic DNA in the samples is detectible. For the investigation, primer designed for genotyping were used. The real-time PCR products were loaded on a gel prepared as described above and were analyzed afterwards.

6.2.7 Cloning of eGFP into pIB/V5-his TOPO/TA

To investigate the interaction of AtPGIP1 with PCOGH28s a binding assay was performed. Cloning of AtPGIP1 in pIB/V5-His TOPO/TA including V5 as native signal have been performed previously in the department followed by successfully expression of AtPGIP1 in Sf9 cells, so that AtPGIP1 could have been used for further studies. In order to realize the binding assay with AtPGIP1 and PCOGH28-1–9, first each of the GH28-proteins had to be expressed in Sf9 cells with eGFP as tag. Previous studies showed no expression of GH28-7 in Sf9-cells, so that this construct haven't been tested. AtPGIP1 was already cloned into the pIB/V5-His TOPO/TA, in frame with the coding sequence of a V5-$(His)_6$ epitope, successfully expressed in Sf9 cells and ready to use for the binding assay. GH28-1,3 and 9 have been already cloned in pIB/V5-His TOPO/TA expressed with eGFP as tag in Sf9 cells and were in stock in the department. The other five GH28s (2,4,5,6,8) samples were already cloned in pIB/V5-His TOPO/TA. First eGFP as tag has to be cloned into samples containing GH28 and the expression of these with eGFP as tag in Sf9 cells had to be confirmed. As positive control known of previous investigations, the binding between BRAPGIP3 and GH28-1 was also tested. BRAPGIP3 was also cloned into the pIB/V5-His TOPO/TA, in frame with the coding sequence of a V5-$(His)_6$ epitope, already successfully expressed in Sf9 cells and ready to use.

eGFP-Amplification

Prior to the expression of GH28s with eGFP as tag, eGFP had to be amplified. eGFP was amplified with $NotI_{HF}$ specific forward primer and SacII specific reverse primer including a stop codon at the end (Table 13). 0.1 ng PCO-GH28-1 including a coding sequence of eGFP was used as template. The PCR was carried out with Q5® High-Fidelity 2X Master Mix. Thermocycling conditions for the PCR was performed as described in the manufacturer's guideline with 62 °C as annealing temperature (Table 14). In order to get enough material, three samples were amplified.

After the amplification, 5 µl PCR product were prepared with 1 µl Loading Dye. The PCR reactions were analyzed on a 1.2 % agarose gel and visualized by ethidium bromide staining. 50 ml TAE-Buffer with 3 µl ethidium bromide was prepared. After running 20 min at 140 V, the results have been analyzed with a gel imaging system. Afterwards the three samples were pooled to one sample and a PCR-Product-Cleanup was realized using DNA Clean & Concentrator™-5 according to the manufacturer's protocol [60]. The concentration of the product was determined photospectrometically with Nanophotometer N 60.

Enzyme-Digestion

Each of the PCO-GH28s (GH28-2,4,5,6,8) and the amplified eGFP were digested with $NotI_{HF}$ and SacII for 2 h at 37° C (Table 3).

Table 3: Setup of eGFP and PCOGH28S for enzyme digestion with $NotI_{HF}$ and SacII

Reagent	***Volume***
eGFP / pIB PCOGH28(*)	1.2 µg / 2 µg (*)
CutSmart Buffer	5 µl
$NotI_{HF}$	2 µl
SacII	2 µl
Nuclease free water	*add to 50 µl*

After the digestion eGFP have been purified with DNA Clean & Concentrator™-5 according to the manufacturer's protocol [60]. In contrast, a gel-cleanup was required for the five PCO-GH28s. The treatment was conducted according to the manufacturer's protocol as described above (6.2.4.2 Real-Time-PCR – Primerspecifity). At the end the samples were eluted with 10 µl nuclease-free water. Concentration of all samples after cleanup was determined with Nanophotometer N60.

Ligation and Transformation

Ligation of eGFP with each of the PCO GH28s was carried out with T4 DNA-Ligase based on the manufacturer's guideline. 3 µl of each reaction have been transformed into 25 µl competent cells. The transformation was realized as described previously (6.2.4.2 Real-Time-PCR – Primerspecifity). But in this experiment, the picked colonies were directly verified by sequencing without a Mini-Prep. Positive clones were verified by Sanger sequencing.

6.2.8 Expression of PCOGH28s in Sf9 cells

Transfection

Sf9 cells were cultivated in Sf-900™ III SFM supplemented with 50 µg/ml Gentamicin until 70 % confluence was reached. Transfection of three positive clones per construct was performed with FuGENE HD as a transfection reagent in 24-well plates [61]. The ratio of FuGENE HD to DNA was 3:1. Amount of cDNA can be calculated by the plate area. The culture medium was harvested after 72 h transfection and stored at 4 °C until use.

SDS-PAGE

Expression of the positive constructs was verified by western blotting. Prior to Western Blot, the proteins were separated by SDS-PAGE (Table 4). The prepared samples were first boiled 5 min at 95 °C and then loaded on the gel. 5 µl PageRuler™ Plus Prestained Protein Ladder was applied as marker. The treatment was conducted 90 min at 120 V and the gel was ready to use for western blotting.

Table 4: Setup for SDS-PAGE reaction

Component	***Volume***
10% SDS	1.2 ml
4x Sample Buffer	3 µl
20x RA	0.6 µl
sample	7.2 µl
Σ	**12 µl**

Western Blot

The prepared gel was washed with water and incubated in Tris/Glycine-Buffer (TG-Buffer). Meanwhile the PVDF membrane was activated with methanol. The membrane, filter paper and fiber pads were soaked in TG-Buffer for a few minutes. A transfer sandwich was assembled with the membrane and gel in the middle, the filter paper in contact with them and the fiber pads on the outside. The transfer cassette was placed in a tank filled with TG-Buffer and the treatment was conducted 30 min at 100 V. All next steps are performed with agitation. The PVDF membrane was blocked in 1x TBS, 0.1 % tween 20 and 5% non-fat dry milk for 1h at room temperature. Afterwards, the membrane was incubated in 1x TBS, 0.1 % tween 20, 5% non-fat dry milk and 1:10000 Anti-GFP antibody over night at 4 °C. Next day, the membrane was washed 3 times 10 min in 1x TBS, 0.1 % tween 20 and once in 1x TBS for a few minutes. ECL solution was added to the membrane separated from the TBS. ECL consists of two different solution: First solution contains 5 ml 100 mM Tris (pH 8.5) and 3 ml H_2O_2, whereas the second solution consists of 5 ml 100 mM Tris (pH 8.5), 50 µl luminol and 22 µl coumaric acid. In a dark room the membrane was placed in a film cassette, and a film was placed on the membrane and revealed. All predicted weights were calculated with EditSeq 15.

6.2.9 Binding assay

Midi-Prep

In order to get enough material for binding assay, a Midi-Prep was performed for a single positive clone per construct of all GH28s, AtPGIP1 and BRAPGIP3. The treatment was carried out following the manufacturer's protocol with some modifications [62]. Precipitation and washing were conducted at 15.000 g, respectively. The air-dried pellet was dissolved in 200 µl water. Concentration determination was realized photospectrometically.

Transfection

Each of the GH28s, AtPGIP1 and BRAPGIP3 were transfected in 6-well plates in the same way as described previously. In this experiment, not only the culture medium of the constructs was harvested, but also the culture medium of non-transfected samples as negative control (C^-).

Binding-Assay

After 72 h transfection and harvesting all samples were ready to use for the binding assay. Due to the fact that AtPGIP1 and BRAPGIP3 has a V5 epitope, Anti-V5 beads were used for investigating the interaction between AtPGIP1 and each GH28. All next centrifugation steps were performed 2 min at 1000 g and 4 °C. First of all, 15 µl V5 beads had to be equilibrated in 150 ml non-transfected cultural medium. Afterwards, eight beads were incubated with 1.5 ml AtPGIP1, one with 1.5 ml BRAPGIP3 and nine with non-transfected cultural medium as negative control in a rotary shaker over night at 4 °C. The suspensions were centrifuged, washed with 150 µl cultural medium and centrifuged again. 500 µl of each GH28 was added to a V5-bead previously incubated with AtPGIP1 and to the respective negative control. The positive control BRAPGIP3 and the negative control of this construct was incubated with 500 µl GH28-1. On the next day, all suspensions were centrifuged and washed two times with 500 µl cultural medium. The interaction of the two proteins was tested by western blotting using 1:10000 anti V5 antibody on one gel and 1:10000 Anti-GFP antibody on another gel. Meanwhile, the expression of the GH28s was proved by western blot with Anti-GFP antibody using the harvested samples from the transfection. Anti-V5 antibody was added to verify the expression of the harvested BRAPGIP3. GH28-1 and AtPGIP1 couldn't be tested since the complete harvested volume were used for the binding assay. All western blots were conducted as described previously.

7. RESULTS

7.1 Genotyping

In order to verify the homozygosity of the used plant lines, a PCR was performed, and the results were analyzed by gel-electrophoresis. The clear bands on the gel confirmed the wildtype Columbia-0 and both homozygous mutant lines (Figure 1). Almost all primer combinations amplified the templates with the expected product size (Table 6, Table 7). Surprisingly, PGIP1-mutant line was also amplified with the last primer combination including T-DNA specific forward primer (FWD) and PGIP2 gene-specific reverse primer (REV) (Figure 1: Primer combination 4 with PGIP1-mutant as template). To verify the mutant line, another PCR was realized using extracted gDNA samples of each line as template and full-length primer (KpnI specific forward and NotI specific reverse primer; Table 8). Gel-electrophoresis results confirmed the right mutant lines (Figure 2). The amplified PCR product is about 1200 bp long (Figure 1: Primer combination 4 with PGIP1-mutant as template), whereas this primer combination should amplify a product with a size of about 480 bp (Table 7: PGIP2).

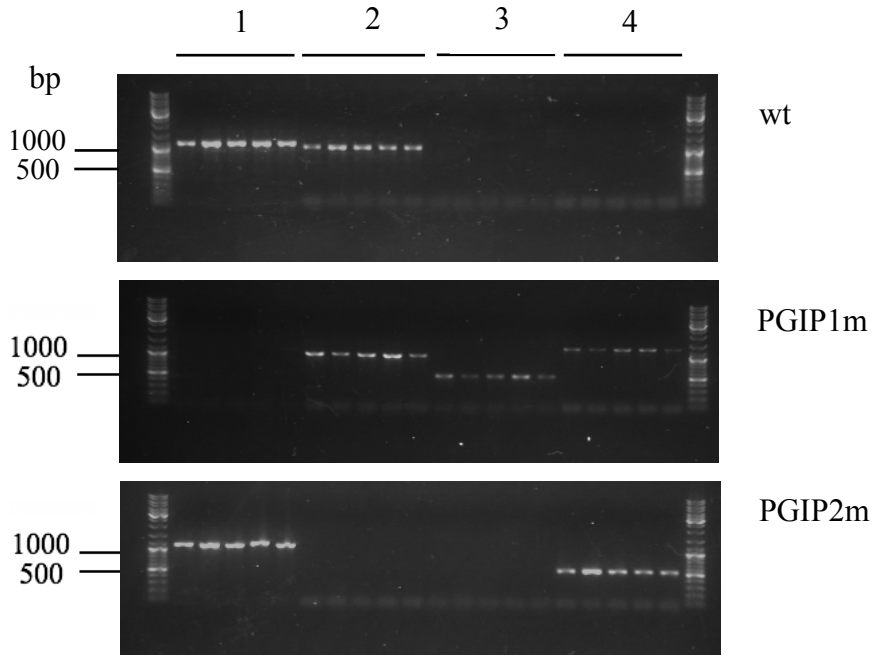

***Figure 1: Genotyping of used plant lines**. Template: wt, PGIP1m, PGIP2m; Primer: PGIP1 gene specific FWD and REV (1), PGIP2 gene specific FWD and REV (2), PGIP1 T-DNA specific FWD and gene specific REV (3), PGIP2 T-DNA specific FWD and gene specific REV (4)*

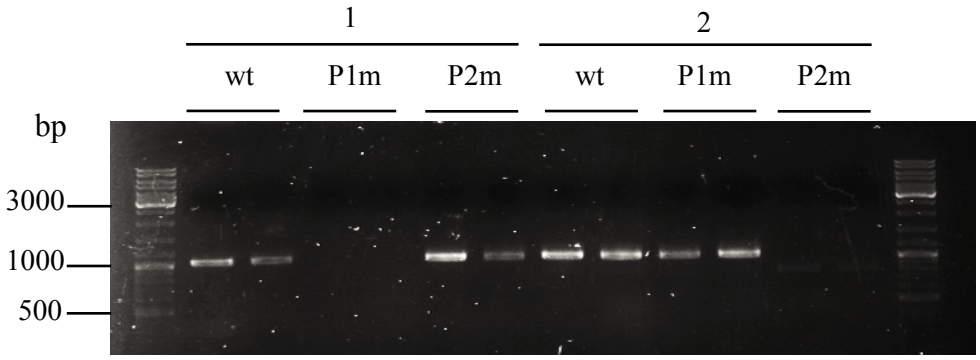

Figure 2: Genotyping PCR with full length primer. *PGIP 1 primer (1), PGIP2 primer (2); gDNA of wt, P1m (PGIP1-mutant) and P2m (PGIP2-mutant) as template*

Since the distance between both genes is only 507 bp [14] and the T-DNA insertion in both knocked out genes is the same, it is possible that the T-DNA specific forward primer binds on the T-DNA in the knocked-out PGIP1 gene. This would also explain the large product size (Figure 14). In general, wildtype plants and the homozygosity of the used mutant lines were determined as recommended by the manufacturer and were ready to use for the treatment assay.

7.2 Real-Time PCR Troubleshooting

The aim of this study was, to investigate the gene regulation of PGIPs in response to feeding by *P. cochleariae* and wounding using the method of real-time PCR. As mentioned before tandemly duplicated, two neighboring PGIP genes were analyzed, which offered some problems until results for real-time PCR could be achieved. In this chapter, the difficulties will be summarized.

Due to the fact that in this case not only wildtype plants were analyzed, but also mutant lines with knock-out of one PGIP gene, it was possible to take each of the mutant line as negative control for each primer combination of the knocked-out gene in real-time PCR since knocked-out genes shouldn't have any transcripts. According to melt curve analysis, the mutant lines indicated a real-time PCR product with the unexpected primer combination, which shouldn't happen since each mutant line possess one PGIP gene and shouldn't be able to get a product with both PGIP gene-specific primer combinations. There could be many reasons for the amplified product. The first idea was performing a PCR using cDNA samples of every line as template and PGIP full-length primer (Table 8) in case of presence of a transcript of the knocked-out gene. Gel electrophoresis results negated this assumption since the mutant lines were not amplified with the knocked-out gene specific primer combination (Figure 3).

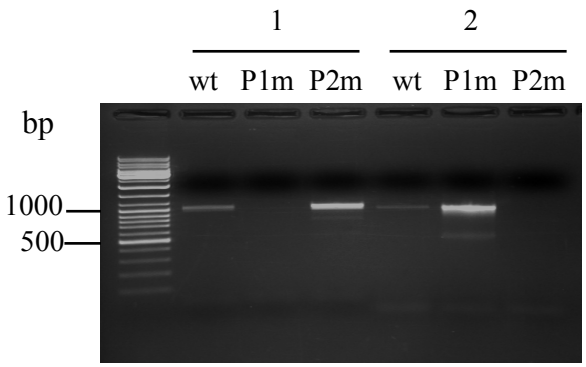

Figure 3: PCR with full length primer. *PGIP1 primer (1), PGIP2 primer (2); cDNA of wt, P1m (PGIP1-mutant), P2m (PGIP2-mutant) as template*

As reported previously, it is known that the two PGIP genes are highly similar, which increases the probability of unspecific real-time PCR primer. In order to check this possibility, the specifity of the real-time PCR primer was verified as described in 6.2.6. Comparing the results from sequencing with the data sequence of each gene confirmed the specifity of both primer (Table 9).

Another reason for detecting the unexpected real-time PCR-product could be the carryover of genomic DNA. During RNA-Extraction with Trizol and the DNase digestion most of the gDNA should have been eliminated, so that it could not be detectible in the real time PCR. To check this assumption, a real-time PCR was realized comparing cDNA and RNA templates with each other (6.2.6 – *Determination of genomic DNA amount in cDNA samples)*. The results showed that RNA samples contained still too much gDNA affecting the real time PCR results since all primer combinations gave a product for all the tested RNA samples. For that reason, the RNA of the collected leaf samples was extracted with InnuPrep RNA Mini Kit including a specific gDNA removing column. To be ensure the gDNA elimination, the method of cDNA synthesis was also changed containing a specific gDNA eraser. Again, a real time PCR was performed using cDNA and RNA as template and comparing these two. To be ensure that the amplified product is genomic DNA, primer designed for genotyping (7.1; Table 7) were used. Loading some real-time PCR products on a gel showed clearly the existence of genomic DNA (Figure 4).

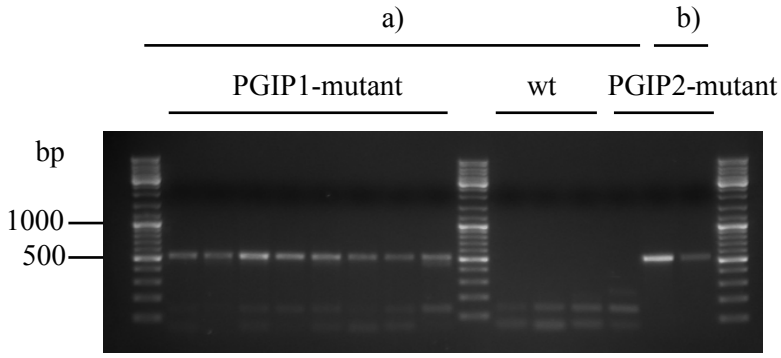

Figure 4: Gel-electrophoresis results of cDNA real-time PCR products amplified with genotyping primer*. a) T-DNA specific FWD / PGIP1 gene specific REV b) T-DNA specific FWD / PGIP2 gene specific REV*

Nevertheless, the amount of genomic DNA in RNA samples was still influencing real-time PCR results. Hence, this time the method of specific mRNA extraction was tested and showed positive effect on eliminating gDNA. Using mRNA samples as templates with the specific real time PCR primer showed very low expression levels not affecting the real-time PCR results. Meanwhile, new primer combinations were also designed, where one of the primer spans the exon-intron boarder, so that only cDNA and not gDNA could be used as template (Table 12). Loading the real-time PCR products on a gel showed unspecific primer amplifying unspecific products (Figure 5), so that these exon-intron boards spanning primer couldn't be used for real-time PCR.

However, the gDNA elimination in mRNA samples was not influencing the results and the real-time PCR primer were specific according to sequencing results, so that the reverse transcribed cDNA's of the extracted mRNA samples and the real-time PCR primer (Table 9) could be used.

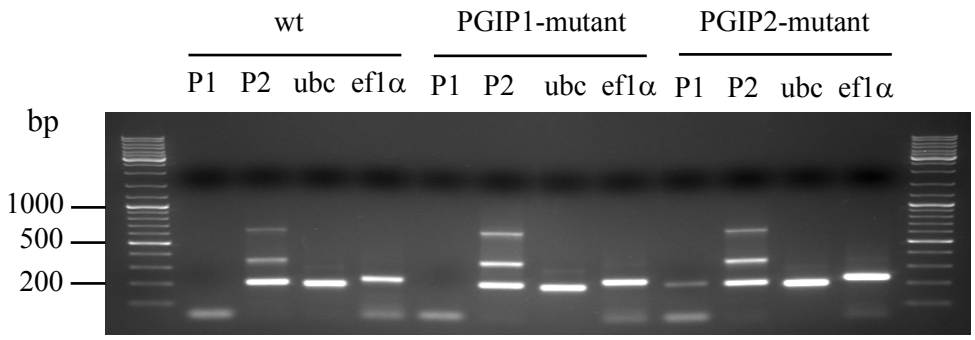

Figure 5: Gel-electrophoresis results of real-time PCR products including one exon-intron boarder spanning primer. *DNA templates are used of wt, PGIP1-mutant and PGIP2-mutant with four different primer combinations: PGIP1 (P1), PGIP2 (2), ubc and efl1α*

7.3 AtPGIP-Expression is regulated by different treatments

For verifying the regulation of both PGIP genes in leaves, a real time PCR was performed using reverse transcribed cDNA samples from three different treatments: Non-treated plants represented control plants with a steady state level of PGIPs. Furthermore, leaves of *A. thaliana* were wounded with a small forceps. Feeding experiments were conducted by putting a mix of *P. cochleariae* larvae and adulti on leaves of *A. thaliana.* All treatments were performed on wildtype and two different mutant lines of *A. thaliana* for 20 hours. PGIP gene-specific primer and, Ubiquitin C (ubc) and Elongation-factor1 alpha (ef1α) as reference genes were used for the amplification (Table 9). Since the results of both reference genes didn't differ from each other, Ubiquitin C was chosen as reference gene for analyzing the real-time PCR results.

An interesting point of view is the influence of different treatments on gene expression, respectively. Comparing the three treatments in every plant line shows an upregulation of PGIP genes by *Phaedon cochleariae* feeding on all plants (Figure 6: A, B, C). All values are statistically significant. PGIP1 and PGIP2 expression is induced by wounding, but it is statistically not significant in wt and PGIP2-mutant (Figure 6: A, B, C). This declares a high induction by consequently attacking plants by *Phaedon Cochleariae* feeding. Generally, feeding in all plant lines shows a statistically induced expression, whereas induced expression by wounding depends on the plant line.

Plant-treatment assay was carried out using not only wildtype plants, but also two mutant lines with knock out of one PGIP gene by T-DNA insertion, respectively. The key question here is, how the mutant lines deal with one knocked-out gene, which may play an important role in plant defense (Figure 7). In wounded plants, there is obviously a significant difference in transcript levels of PGIP1. The PGIP1 expression is much higher in wt than in the mutant line. However, the PGIP2-mutant tries to compensate the knocked-out gene during *Phaedon Cochleariae* feeding on *A. thaliana* plants (Figure 7: A). In this case, the gene expression of PGIP1 increases extremely in comparison to the one in the wt plant line. The same expression pattern can be found for PGIP1 expression in control plants. In both cases, the expression of PGIP1 is about 2-fold higher in mutant lines compared with wt.

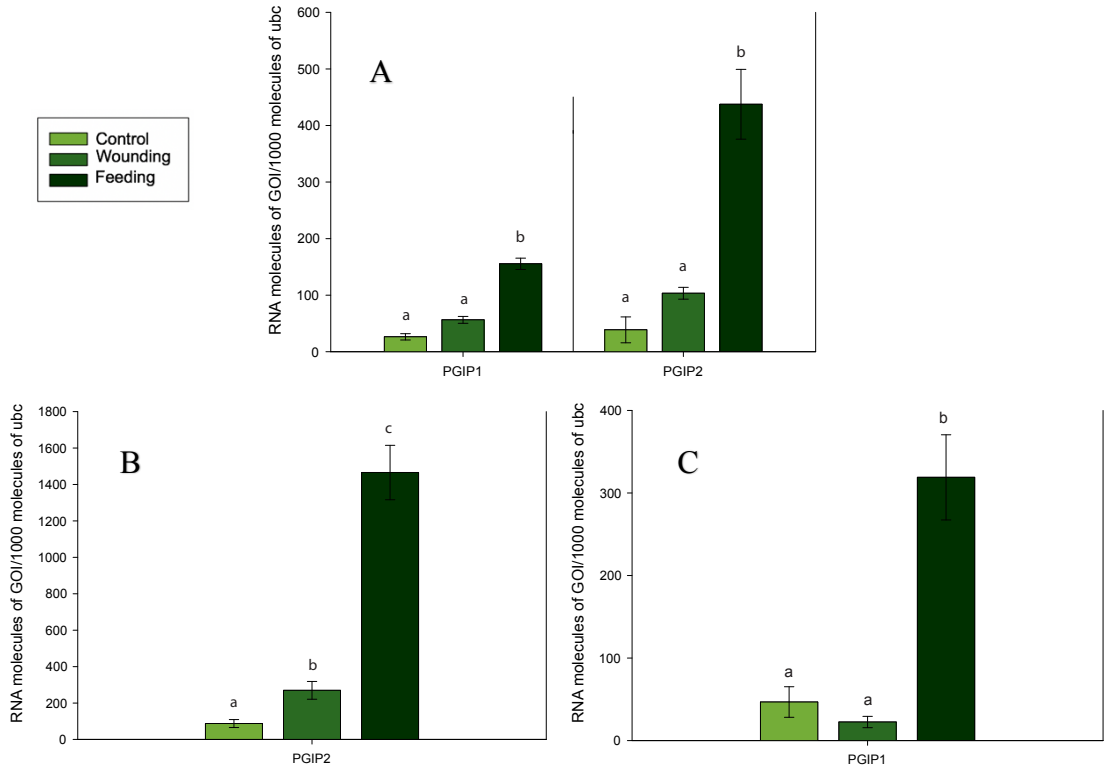

Figure 6: Real-Time PCR results: Comparing PGIP gene expression based on the treatment of Arabidopsis thaliana. *Transcript levels are expressed as RNA molecules of GOI/1000 molecules of Ubiquitin C (ubc) as reference gene in three mutant lines; (A) wildtype (B) PGIP1-mutant (C) PGIP2-mutant; values are determined as mean ± SD*

Furthermore, transcripts coding PGIP2 are statistically the same in wt and PGIP1-mutant for control plants, while PGIP2 transcripts in wounded and fed plants were statistically more expressed in PGIP1-mutant than wildtype (Figure 7: B). The statistically highest transcript level was expressed in fed PGIP2-mutant line. Remarkably, the expression of PGIP2 in the mutant plant line is about 4-fold higher than in wt in response to feeding.

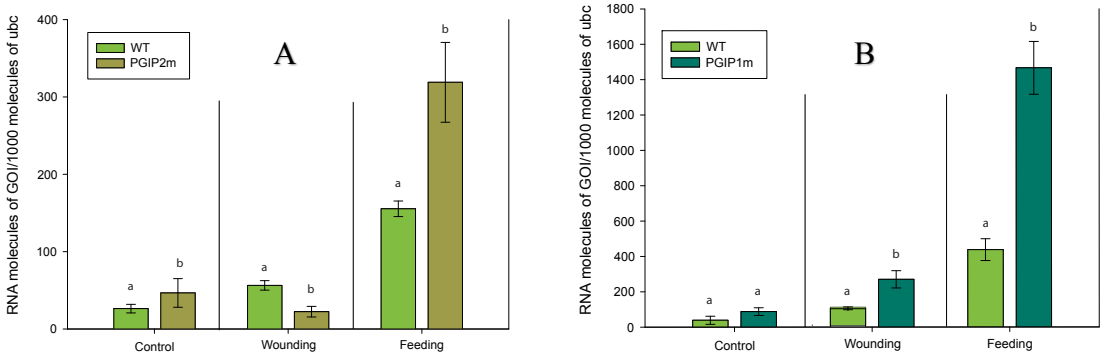

Figure 7: Real-Time PCR results: Comparing PGIP gene expression based on the plant line of Arabidopsis thaliana. *Transcript levels are expressed as RNA molecules of GOI/1000 molecules of Ubiquitin C (ubc) as reference gene in three mutant lines; (A) PGIP1 Expression in wt and PGIP2m based on the treatment (B) PGIP2 Expression in wt and PGIP1m based on the treatment; values are determined as mean ± SD*

In general, it seems likely that both mutant lines compensate the knocked-out gene by an induced expression of the other PGIP gene in case of feeding by *P. cochleariae.* Compensation of PGIPs in wounded plants is only present in the PGIP1-mutant line, PGIP2-mutant line doesn't show an increased expression. In control plants PGIP2-mutant line tries to compensate the knocked-out gene, whereas PGIP1-mutant line doesn't show a statistically difference to the wt plant.

Another interesting view is the expression of PGIP1 and PGIP2 in wildtype (Figure 8). Here, the interesting study is, if the expression of both genes differs from each other based on the treatment. The expression pattern of PGIP1 and PGIP2 in control plants doesn't show a statistical difference. However, in wounded and fed plants PGIP2 expression is higher than PGIP1, which means that both genes have a different reaction to the respective treatment. Remarkably, AtPGIP2 expression is about 2-fold higher than AtPGIP1 expression in response to feeding and wounding in wildtype plants. The upregulation of PGIP2 gene is much higher by *P. cochleariae* feeding on *A. thaliana* than by wounding the plants. Generally, the expression of PGIP1 and PGIP2 doesn't show a statistically difference in control plants, whereas treatment by wounding or *P. cochleariae* feeding increases the expression of PGIP2 enormously. It seems very likely, that both genes are regulated in two different ways, which react differentially to biotic and abiotic stress stimuli, respectively.

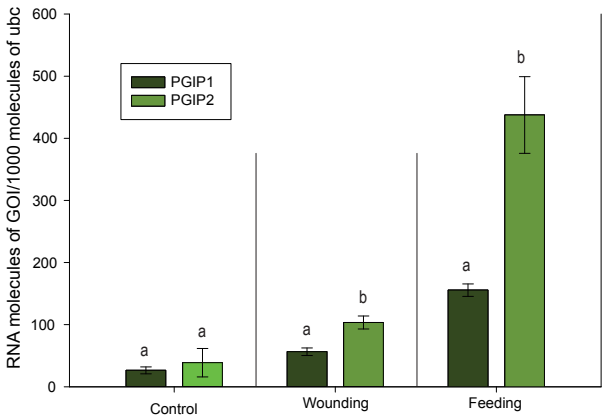

Figure 8: Real-Time PCR results: Comparing gene expression of PGIP1 and PGIP2 in wildtype of Arabidopsis thaliana. *Transcript levels are expressed as RNA molecules of GOI/1000 molecules of Ubiquitin C (ubc) as reference gene based on three different treatments: Control, wounding, feeding*

7.4 Interaction of AtPGIP1 with PCOGH28s

Another aim of this study was, to investigate the interaction of PGIP1 – including V5 as native signal – in *A. thaliana* (AtPGIP1) with GFP-taged PGs of *P. cochleariae* (PCOGH28s). The cloning of AtPGIP1 into pIB/V5-His TOPO/TA and the successfully expression in Sf9-cells was realized previously in the department. It is well-known, that *P. cochleariae* encodes nine PGs [22], whereby previous studies showed no expression of PCOGH28-7 in Sf9-cells, so that this construct haven't been tested. GFP-taged GH28-1, -3 and -9 were already successfully cloned in pIB/V5-His TOPO/TA and were ready to use for the binding assay. The other GH28s were already cloned into pIB/V5-His TOPO/TA, but didn't have eGFP as tag, so that first eGFP has to be amplified and cloned into the plasmids containing the respective PCOGH28.

After successfully amplifying eGFP with $NotI_{HF}$ specific forward and SacII specific reverse primer (Figure 9) including a stop codon, eGFP was cloned in pIB PCOGH28s and transformed using TOP10 cells.

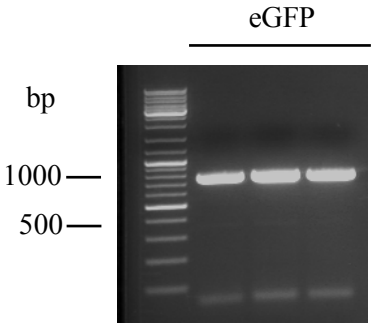

Figure 9: eGFP-Amplification with $NotI_{HF}$ specific forward primer and SacII specific reverse primer.

Three positive clones per construct were transfected in Sf9-cells, and the expression was tested by Western blot using Anti-GFP antibody. Western blot analysis showed successful expression of all GH28s with GFP as tag in Sf9 cells (Figure 10). All GH28s are in a range of 38 kDA to 40 kDA heavy. eGFP has a mass of about 26.9 kDA. According to this information, all bands on the gel confirm the expression of each GH28 with eGFP as tag. As positive control, previously successful expressed PCOGH28-1, -3 and -9 were used. As expected, the negative control shows no expression. Surprisingly, bands below the expected weight can be also found on the gel, which can be caused for example by the high sensitivity of the anti-GFP antibody or partial degradation of the proteins. In general, all GH28 constructs with eGFP as tag were expressed successfully and can be used for investigating the binding between GH28s and PGIP.

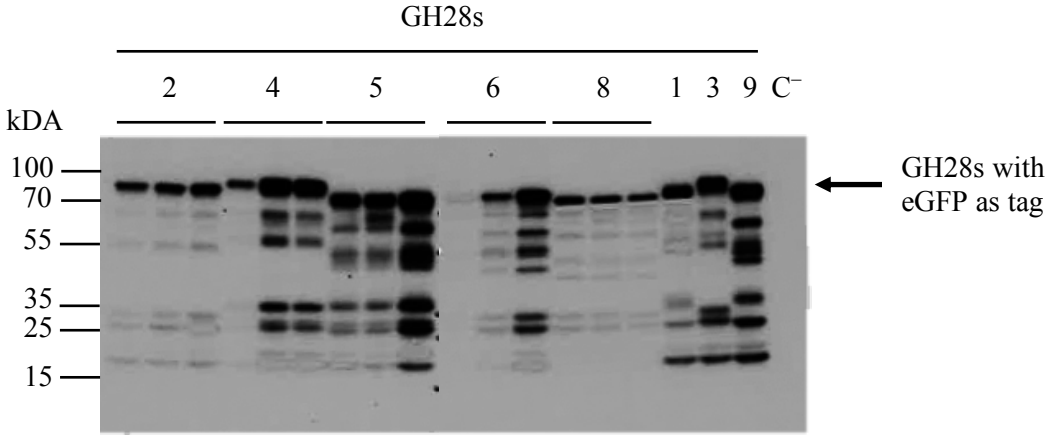

Figure 10: GH28s expression with eGFP tag in Sf9 cells*. Western Blot results using Anti-GFP antibody and three positive clones per construct; From left to right: Investigated samples: GH28-2, GH28-4, GH28-5, GH28-6, GH28-8, Positive Control: GH28-1, GH28-3, GH28-9, Non-transfected sample as negative control C–*

The interaction of AtPGIP1 including V5 as native signal and GH28s with eGFP as tag should be analyzed. Previous studies in the department showed a very likely interaction of BRAPGIP3 with PCOGH28-1, so that this construct was used as positive control.

For the binding assay V5 beads were used. The binding of V5 beads to the PGIP is required for the binding assay. To be ensure that AtPGIP1 and BRAPGIP3 have bound to the V5 beads, a western blot was realized using Anti-V5 antibody. The clear bands on the gel confirm the binding between the PGIPs and V5 beads (Figure 11). The predicted weight of AtPGIP1 was 37 kDA, with the V5-tag adding ~1.4 kDa for a total of ~40 kDA. Based on Western Blot results, the detected protein band was consistent with the predicted molecular weight. GH28s alone incubated with V5 beads as negative control indicate that the GFP-taged GH28s cannot bind to the V5 beads and that the Anti-V5 antibody cannot detect the GFP-taged GH28s.

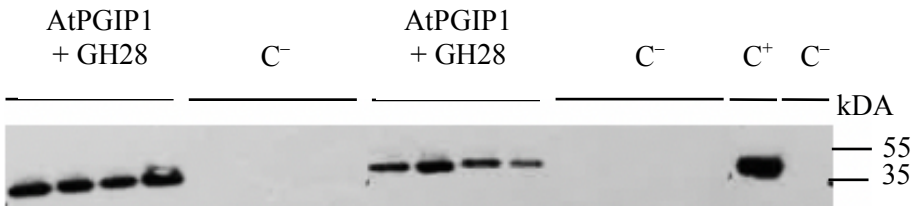

Figure 11: Binding of AtPGIP1 to V5 beads. *Western Blot analysis using Anti-V5 antibody; During the binding assay V5 beads were incubated with AtPGIP1 and non-transfected samples representing the negative control. GH28s were added to all beads, which were incubated with AtPGIP1, and to all beads without AtPGIP1, so that each GH28 had a negative control (C–). BRAPGIP3 (C+) was used as positive control. From left to right: AtPGIP1 + GH28-1,3,2,4 followed by the respective negative control; AtPGIP1 + GH28-5,6,8,9 followed by the respective negative control; BRAPGIP3 + GH28-1 followed by the respective negative control*

In order to check the binding between PGIPs and GH28s a western blot was performed using Anti-GFP antibody. As described above the predicted weight for GH28s with eGFP as tag is ~70 kDA. The clear bands on the blot show the binding of all GH28s, except for GH28-6 and -8 (Figure 12). Interestingly, the same bands with the same mass can be also found in the negative control. As described above, the unspecific binding of GH28s to V5 beads is excluded. On account of that, the binding between PGIPs and GH28s cannot be absolutely confirmed. The bands for the negative control indicate a possible problem of the conditions during the binding assay such as inaccurately washing of the beads, which had to be optimized. It cannot be clearly claimed whether the bands are caused due to the binding of GH28s to PGIPs or due to some inexactness in the binding assay.

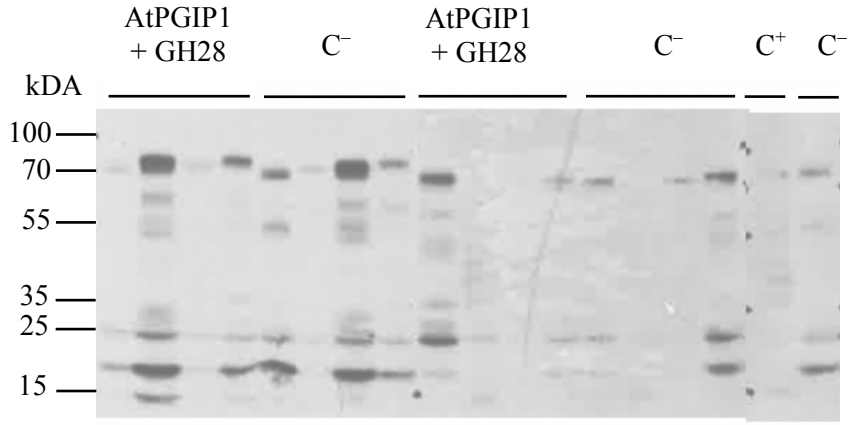

Figure 12: Binding of GH28 to AtPGIP1. *Western blot analysis using Anti-GFP-antibody. During the binding assay V5 beads were incubated with AtPGIP1 and non-transfected samples representing the negative control. GH28s were added to all beads, which were incubated with AtPGIP1, and to all beads without AtPGIP1, so that each GH28 had a negative control (C^-). BRAPGIP3 (C^+) was used as positive control. From left to right: AtPGIP1 + GH28-1,3,2,4 followed by the respective negative control: GH28-1,2,3,4; AtPGIP1 + GH28-5,6,8,9 followed by the respective negative control: GH28-5,6,8,9; BRAPGIP3 + GH28-1 followed by the respective negative control: GH28-1*

Meanwhile, another western blot was carried out with the harvested samples after 72 h transfection to be ensure the expression of the samples used for binding assay. The expression of the GH28s and BRAPGIP3 was successfully according to the Western Blot results (Figure 13). The predicted mass for GH28s with eGFP as tag and for BRAPGIP3 with V5 tag are described above. GH28s shows an overexpression in comparison to BRAPGIP3. But comparing BRAPGIP3 expression before (Figure 13) and after the binding assay (Figure 11), the expression of BRAPGIP3 is highly enriched.

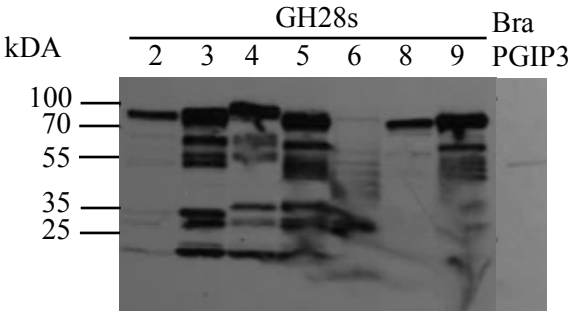

Figure 13: GH28s expression with eGFP tag and BRAPGIP3 expression with V5 tag in Sf9 cells*. Western Blot results using harvested samples after transfection; For PCOGH28s Anti-GFP antibody and for BRAPGIP3 Anti-V5 antibody was used. From left to right: GH28-2,3,4,5,6,8,9 and BRAPGIP3*

8. DISCUSSION and OUTLOOK

8.1 Real-time PCR Troubleshooting

The difficulties achieving evaluable data with real-time PCR have been described in 7.1. As mentioned before, both mutant lines with knock out of one AtPGIP represented a negative control for real-time PCR. In an unexpected way, cDNA of mutant lines used as a template showed PCR products with the knock-out corresponding gene specific primers. Investigating the primer specify and the amount of gDNA in RNA samples were required for searching the cause of the amplified product of the negative control. It is well-known that both AtPGIPs are tandemly located with a difference of 507 bp on Chromosome 5 [14]. The short distance of the two genes from each other increases the probability of unspecific primer. Genotyping results are a significant example for this (Figure 1). Therefore, the specifity of the primer has to be investigated accurately.

Furthermore, the detectible amount of genomic DNA caused trouble. In this study, treatment assays were carried out on leaves of Arabidopsis. Previous studies showed low expression of PGIPs in leaves compared to the expression in roots [35, 63]. Genes of interest were amplified at late cycle number in control plants, which confirmed low steady state expression of PGIPs in leaves. This underlines the importance of eliminating genomic DNA in RNA samples since even little amount of gDNA might have a big impact on the data.

AtPGIP1 possesses an intron with 69 bp and AtPGIP2 one with 83 bp [14]. This is not really usual due to the fact that most PGIP genes are intronless [25]. However, the short introns in both genes seemed like to be an advantage for designing exon-intron boarder spanning primer amplifying only cDNA and not gDNA. Nevertheless, as mentioned above the trouble with unspecific primer occurred, so that this advantage could not be useful here (Figure 5).

At the end, the amount of detectible genomic DNA was eliminated through mRNA enrichment. It should be clearly pointed out that the amount of the genomic DNA has to be investigated for evaluable data. Interestingly, this troubleshooting occurred since the primer matching the knocked-out gene with the corresponding cDNA of the mutant line were used as negative control. If in this study only wildtype plants would have been investigated, the amplification of the genomic DNA might not have been recognized. This leads to the conclusion, that a significant negative control plays an important role for reliable data.

8.2 Gene expression analysis of AtPGIPs

8.2.1 Different gene regulation based on the treatment

In this study, it has been proved that AtPGIP expression is upregulated in response to different treatments (Figure 6). AtPGIP1 and AtPGIP2 are both upregulated in response to feeding by *Phaedon cochleraiae*. The highest level of transcripts shows AtPGIP2 expression in PGIP1-mutant line (Figure 6: B). The induced expression in response to feeding is present in all plant lines. The same expression pattern indicated BnPGIP1 in response to feeding by a flea beetle. Here, in this case BnPGIP2 was not induced by beetle feeding [35]. In sugar beet, all three BvPGIPs were also upregulated in response to beetle feeding [63]. However, feeding by *P. rapae* on Arabidopsis didn't show an induced expression [64].

Compared to feeding by *Phaedon cochleariae* wound induced expression is only statistically significant for AtPGIP2 expression in PGIP1-mutant line (Figure 6: B), the other two wounded plant lines don't show a statistically upregulation, even if they have a higher transcript level than the control plants. Interestingly, a previous study declared an induced expression of both PGIP genes in Arabidopsis 48 h after wounding of wildtype plants [14]. In Medicago *truncatula* MtPGIP1 transcripts accumulated rapidly, whereas this pattern couldn't be found for MtPGIP2 [65]. Furthermore, wounding induced the expression in bean PvPGIP1 and PvPGIP2 and also in BnGIP1 and BnPGIP2 [35, 40]. Surprisingly, the maximum of induced expression by wounding was achieved in PvPGIP1 after 3 h and in BnPGIPs after 2 h. At 24 h the expression pattern decreases extremely. In panax ginseng mechanical wounding proved impact on transcript level [66], whereas in strawberry the PGIP was clearly not induced by wounding [67]. So, previous studies confirmed many times the upregulation of PGIPs in response to wounding, whereas here only one plant line shows an upregulation in response to feeding. In this study, leaves of A. thaliana were wounded with a small forceps a few times in a total incubation time of 20 h. As mentioned before, Ferrari et al. have proved an induced expression of both PGIPs in Arabidopsis. Nevertheless, they could show this pattern in 48 h wounded plants [14], whereas in this study leaves were collected after 20 h. Furthermore, it has been proved that e.g. BvPGIPs of sugar beet are 3.8 – 5.4 higher expressed in roots than in leaves [63]. The same pattern can be also found in BnPGIPs [35]. Here, leaves of Arabidopsis were wounded and collected for the analyses. Since the expression of PGIPs are less in leaves than in roots, it might be worth considering

collecting the wounded leaves after a longer incubation time, e.g. 48 h as realized by Ferrari et al. Additionally, due to the fact that in some cases as described above the maximum of induced expression is achieved after a few hours, the leaves could be also collected after a short time, e.g. 3 h or 6 h. Feeding Arabidopsis leaves by *P. cochleariae* was a consequently process, whereas wounding with a small forceps was performed from time to time (6.2.2 Plant-Treatment-Assay). It seems very likely, that the damage caused by wounding was lower and differed from that by beetle feeding. As a consequence of that, the number of mechanically wounding could be also increased to have two comparable treatments regarding to the quantity and quality. In general, the results show that PGIPs play an important role in plant defense since they are highly expressed in response to biotic and abiotic stress stimuli. Feeding by *P. cochleariae* leads to a higher upregulation than wounding, which might be caused by consequently attacking the plant. For further studying, the treatment assay should be repeated with some modifications as described above.

8.2.2 AtPGIP1 and AtPGIP2 expression in wt

In this study, the wildtype plants were also analyzed regarding the expression of both AtPGIP genes (Figure 8). It is well-known, that AtPGIP1 and AtPGIP2 are regulated in response to different signal pathways: Ferrari et al. showed that PGIP1 responds – among others such as fungal infection, feeding and wounding – to oligogalacturonide elicitors, whereas PGIP2 is upregulated by jasmonide [14]. Nevertheless, the expression of both genes didn't differ in their experiments. This pattern can be also found for control wildtype plants, where the expression of both genes doesn't show a statistically difference (Figure 8). Biotic or abiotic stress such as feeding, or wounding increases obviously the expression of both AtPGIPs as mentioned before. AtPGIP2 is upregulated much higher than AtPGIP1 in response to both treatments. Remarkably, previous study showed the same expression pattern of AtPGIP1 and AtPGIP2 in response to wounding, whereas in this study AtPGIP2 is upregulated 2-fold higher than AtPGIP1. Such differential expression patterns were also obtained when *B. napus* were mechanically wounded, respectively. In that case, BnPGIP1 was higher expressed than BnPGIP2 [35]. The differential expression of both genes could be caused by different breeding and leaf conditions, age of the plants or by the wounding intensity.

Many studies showed that duplication and diversification of PGIP gene could result in difference gene regulation, e.g. the four PvPGIPs in bean [40]. Furthermore, previous

studies showed that the JA pathway is induced in response to wounding and tissue-damaging [68], which could also explain the higher upregulation of AtPGIP2 due to the fact that AtPGIP2 responds to jasmonide. In general, it seems very likely, that different signal pathways responses on a different way to biotic and abiotic stress stimuli.

8.2.3 Comparing AtPGIP expression in wt and mutant line

All treatment assays were performed on wt plants as well as on two mutant lines. As described above the mutant lines showed a big advantage in representing a negative control, respectively (8.1). Choosing mutant lines should show the gene regulation of the plant line with one knocked out gene. A key question is, how the mutant line will react to the biotic and abiotic stress stimuli with one missing gene, which actually has a part in plant defense (Figure 7). PGIP2-mutant line tries to compensate the knocked-out gene in control and fed plants by a higher induced expression of PGIP1, whereas in wounded plants the expression is less compared with wt. PGIP1-mutant lines indicates a compensation in control plants. Wounding and feeding treatments show a higher expression of PGIP2 in PGIP1-mutant line than in wt. Both mutant lines fend the plant cell wall stronger than the wt in case of feeding by *Phaedon cochleariae*. This study may help understand how mutant lines are able to recognize a pathogenicity factor and react against that with a higher PGIP expression than the wt plants. The absence of one PGIP may be related to a stronger defense against pathogens. In conclusion, the results indicate that mutant plant lines can be an effective strategy to fend off pathogen attacks, especially off insects.

8.3 Interaction of AtPGIP1 with PCOGH28s

Not all species of insects are known to encode polygalacturonases (PGs). Kirsch et al. showed that nine PGs belonging to GH28 family are produced by *Phaedon cochleariae* (PCOGH28) [22]. The activity of these PGs was confirmed successfully. The key question here is, if AtPGIP1 shows an inhibitory activity against these plant cell wall degrading enzymes. Inhibition of active insect PGs by PGIPs is rarely studied and not all investigations could show the inhibitory activity of PGIPs. It is well-known, that PvPGIP3 and Pvpgip4 from bean inhibited two mirid bugs, *Lygus rugulipennis* and *Adelphocoris lineolatus* [40], whereas PGIPs of Arabidopsis [41] and soybean [42]

couldn't inhibit these mirid bugs. Furthermore, a citrus' PGIP was able to inhibit PG from *D. abbreviatus* [43].

In this study, the interaction of PCOGH28s by AtPGIP1 was investigated with a binding assay. All conditions were fulfilled for studying the PG-PGIP interaction: PCOGH28s were successfully expressed in Sf9 cells (Figure 10, Figure 13). It has been clearly shown that AtPGIP1 bind to V5 beads, whereas an unspecific binding of GFP-taged GH28s to V5 beads could be completely excluded (Figure 11).

Based on the western blot results it cannot be clearly concluded, if the detected bands are caused by the binding of PG to PGIP or by the inexactness of the assay (Figure 12). The possibility cannot be excluded that the samples weren't washed accurately, which might be the reason for the bands related to the negative control. To optimize the assay, changing the washing solution including salt may be a possibility.

Western Blot was performed using not only the samples from binding assay, but also the samples directly harvested after the transfection in order to check the expression. Comparing BRAPGIP3 before (Figure 13) and after the binding assay (Figure 11), the different intensity of the bands shows a poly enrichment of BRAPGIP3 after the binding assay, whereas PCO GH28s were highly abundant on both gels. According to this information, a binding assay could be performed the other way around using GFP beads. In this case, first PCO GH28s would bind to the beads followed by a possible binding of PGIPs to PGs. Bands for the negative control might be less strongly in case of a PG-PGIP interaction since PGIPs might be enriched because of the binding. In general, binding assay can be carried out with another wash solution or with GFP-beads as described above. Further investigations on the interaction between AtPGIP1 and PCOGH28s are required to evaluate the inhibitory activity of AtPGIP1 against insects' PG since PGs play an important role during pathogenesis and might lower the nutritional value of the beetles´ diet.

9. SUMMARY

After 350 million years of coevolution plants have established mechanisms to defend themselves against microbial invasion. PGIPs have been known for 50 years to be an important player in plant defense, which can be found almost in all plant species. It is well-known that they can be upregulated in response to biotic and abiotic stress stimuli such as wounding, feeding and fungal infection, but also show an inhibitory activity against primarily fungi. There is no direct evidence of the inhibition of insect PGs since the inhibition test are conducted with crude extracts and not pure proteins.

In this study, three *A. thaliana* plant lines were used including the Ecotype Col-0 with two PGIP genes and two mutant lines with knock-out of one PGIP gene by T-DNA insertion, respectively.

One goal was, to investigate the gene regulation of *A. thaliana* in response to mechanically wounding and feeding by *P. cochleariae*. Real Time PCR results confirmed an upregulation of both PGIP genes in response to feeding. All plant lines showed this upregulation. Wound induced expression was only statistically significant for PGIP2 expression in PGIP1-mutant line, which shows, that wound induced expression is dependent on the plant line. Comparing both treatments declares that feeding by *P. cochleariae* activates a higher induced expression than mechanically wounding, which means that consequently attacking the plant has a stronger influence on the gene expression than attacking from time to time such as in case of mechanically wounding.

Another interesting view was, how the one knocked-out gene in both mutant lines influence the gene regulation of the other PGIP. Both mutant lines have been compared with the wildtype plant line and it has been successfully shown that in case of feeding by *P. cochleariae* both mutant lines try to compensate the knocked-out gene by a higher induced expression. Depending on the plant line control and wounded plants show also an upregulation. These results indicate that mutant lines may be related to a stronger defense against pathogens and herbivores.

PGIP1 and PGIP2 expression in the wildtype plant was also another interesting key question since it is well-known that they are both regulated in two different signal pathways. It has been successfully shown that the expression of both genes differs in case of biotic and abiotic stress, which indicates differentially regulation of the two signal pathways.

The second aim of this study was to investigate the interaction of PGIP1 from *A. thaliana* and PGs in the gut of *P. cochleariae* (PCOGH28s). It has been successfully shown that PCOGH28s can be expressed with eGFP tag in Sf9 cells. Furthermore, the interaction of PGIP and PG was tested by realizing a binding assay. In regard to western blot results it cannot be clearly claimed whether the PGIPs interact with PGs since the negative controls show also bands with the predicted weight for the interaction of PGIP with PG which leads to the conclusion of a possible inexactness of the binding assay. For further investigations, the binding assay has to optimized as described above, which is required since the inhibition of PGs (as virulence factor) by PGIPs play an important role in plant defense.

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11. APPENDIX

Table 5: PCR conditions for genotyping

Step	***Temperature***	***Time***
Initial Denaturation	95 °C	30 s
Annealing (35 Cycles)	95 °C	20 s
	50 °C	30 s
	68 °C	1 min 20 s
Final Extension	68 °C	5 min
Hold	4 °C	∞

Table 6: Sequences of the gene-specific-primer

Gene	***Sequence 5'-3'***	***Product Size***
PGIP1		1140
Forward Primer	TGACGATTTGAGAAATTTGGG	
Reverse Primer	GGTGACTTGCCGTATCTTGAG	
PGIP2		1025
Forward Primer	CAACAAGTGTTTGTGTGGTGC	
Reverse Primer	ATGGAACCAGAGAGGTCATTG	

Table 7: Sequences of the gene- and T-DNA-specific Primer

Gene	***Sequence 5'-3'***	***Product Size***
PGIP1		525
Forward Primer	GTGGATTGATGTGATATCTCC	
Reverse Primer	CGTTACCTAAAATCTTGGCTCTTG	
PGIP2		480
Forward Primer	ATAATAACGCTGCGGACATCTACATTTT	
Reverse Primer	CGAGTTTACGTAATGAAGAGAGGG	

Table 8: PGIP1 and PGIP2 full-length primer

Gene	Sequence 5'-3'	Product Size
PGIP1		
KpnI Forward Primer	ATTAGGTACCTAAAGATCTCTGTAACCA	997
NotI Reverse Primer	ATTAGCGGCCGCACTTGCAAATTTCAAGAGG	
PGIP2		
Kpn I Forward Primer	ATTAGGTACCTAAAGATCTCTGTCATAAAG	997
Not I Reverse Primer	ATTAGCGGCCGCACTTGCAACTAGGAAG	

Table 9: Real-time PCR primer

Gene	Sequence 5'-3'	Product Size
PGIP1		
Forward Primer	TTGTGTCTCTTGTTCTTGTTCACA	212
Reverse Primer	ATGGTTAAGGCGGTAACACG	
PGIP2		
Forward Primer	CTGTTCTTGCTCTTGTCCACTCTC	224
Reverse Primer	TCGCCGTCTTGTATGATTAGG	
ef1α		
Forward Primer	AGCACGCTCTTCTTGCTTTC	191
Reverse Primer	TCCCTCGAATCCAGAGATTG	
ubc		
Forward Primer	TGGACCGCTCTTATCAAAGG	197
Reverse Primer	CAAGCAGGACTCCAAGCATT	

Table 10: Real-Time PCR – Layout for primer efficiency determination

		Dilution			
		1	2	3	4
Primer	PGIP1	100 %	20 %	10 %	2 %
		100 %	20 %	10 %	2 %
	PGIP2	100 %	20 %	10 %	2 %
		100 %	20 %	10 %	2 %
	ef1α	100 %	20 %	10 %	2 %
		100 %	20 %	10 %	2 %
	ubc	100 %	20 %	10 %	2 %
		100 %	20 %	10 %	2 %

Table 11: Real Time PCR – 96-well-plate layout for each treatment

		Plant line as cDNA template								
		1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***
Primer	***PGIP1***	wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m
		wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m
	PGIP2	wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m
		wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m
	ef1α	wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m
		wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m
	ubc	wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m
		wt	wt	wt	PGIP1m	PGIP1m	PGIP1m	PGIP2m	PGIP2m	PGIP2m

Table 12: Real-time PCR primer combinations including one exon-intron boarder spanning primer

Gene	***Sequence 5'-3'***	***Product Size***
<u>PGIP1</u>		
Forward Primer	GCAGGAACAAACTTACAGGTTCC	275
Reverse Primer	TAGACCAGGTTGTTTTGTTGGAAC	
<u>PGIP2</u>		
Forward Primer	TAGGAACAAGCTTACAGGTCCG	290
Reverse Primer	ACGATCCATGTTGTTTTTTTAGCTCC	
<u>ef1α</u>		
Forward Primer	GTAACAAGATGGATGCCACCAC	219
Reverse Primer	CTTGGGCTCGTTGATCTGGT	
<u>ubc</u>		
Forward Primer	TGCAACCTCCTCAAGTTCGATTC	271
Reverse Primer	AGAAGATTCCCTGAGTCGCAG	

Table 13: eGFP amplification with NotI$_{HF}$ and SacII specific primer

Gene	Sequence 5'-3'	Product Size
PGIP1		
NotI$_{HF}$ Forward Primer	TAATGCGGCCGCTCTGCAGTGAGCAAGGG CGAGG	728
SacII Reverse Primer	TAATCCGCGGTTACTTGTACAGCTCGTCC	

Table 14: PCR conditions for eGFP amplification

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec
35 Cycles	98 °C	10 sec
	62 °C	20 sec
	72 °C	20 sec
Final Extension	72 °C	2 min
Hold	4 °C	∞

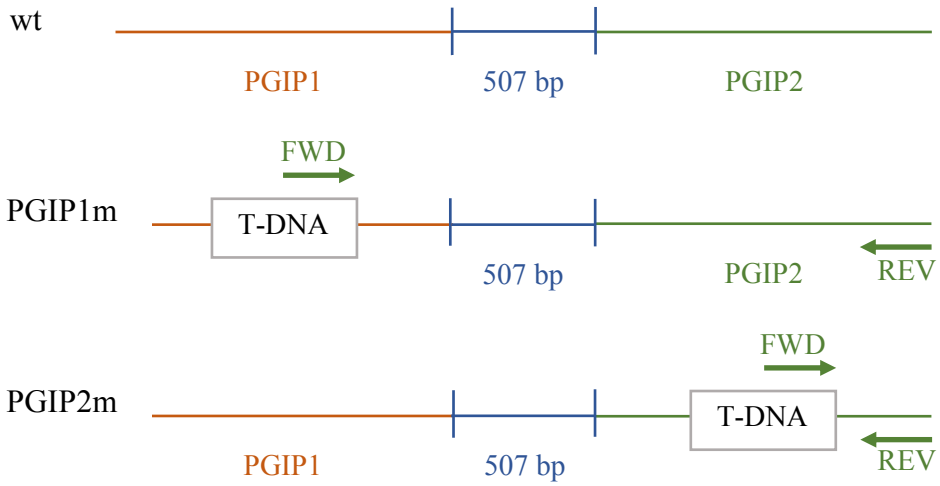

Figure 14: Genotyping – Possible PCR-product amplification. *Col-0 (wt) plant line encodes both PGIP genes, which have a short distance of 507 bp from each other. PGIP1-mutant (PGIP1m) has a T-DNA insertion for PGIP1 gene and encodes only PGIP2 gene. PGIP2-mutant (PGIP2m) has a T-DNA insertion for PGIP2 gene and encodes only PGIP1 gene. The T-DNA insertion in both mutant lines are the same, so that it is possible that the T-DNA specific forward primer for PGIP2 (FWD) gene binds on the T-DNA of PGIP1 gene, which could give a product together with PGIP2 gene specific reverse primer (REV)*

12. DECLARATION OF INDEPENDENT ASSIGNMENT

Ich erkläre hiermit, dass ich die vorliegende Diplomarbeit selbstständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt habe. Diese Arbeit lag in gleicher oder ähnlicher Weise noch keiner Prüfungsbehörde vor und wurde bisher noch nicht veröffentlicht.

Esma Vurmaz
Jena, den 25.09.2018

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