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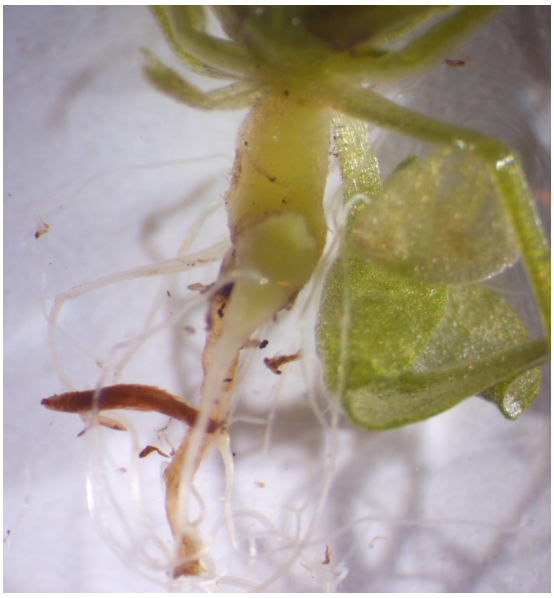
Screening procedures and evaluation of mutant responses

Sytske Drost



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The Arabidopsis - *Plasmodiophora brassicae* interaction: screening procedures and evaluation of mutant responses

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Abstract

Clubroot, caused by the obligate biotrophic protist *Plasmodiophora brassicae*, is an important disease of Brassica oil and vegetable crops, and causes an estimated production loss of 10% worldwide. Infected root tissues develop swollen roots (clubroots) leading to wilting, stunting and premature ripening of the aboveground organs. During infection, *P. brassicae* interferes with the metabolism and hormone homeostasis of the host to avoid plant defense responses.
The aim of this thesis was to study the role of several host genes in the disease response in an *Arabidopsis thaliana – P. brassicae* infection system. The investigated genes are involved in brassinosteroid (BR) biosynthesis and signaling, the jasmonic acid (JA) and salicylic acid (SA) defense pathway, and in lipid transport. BRs are plant hormones essential for cell expansion and elongation. GUS-reporter strains of *A. thaliana* revealed an increased expression of BR signaling and biosynthesis genes in clubroots. We showed that BR signaling and biosynthesis in the host might play an important role for the disease development in infected tissues. Roots infected with *P. brassicae* contained a high amount of lipid bodies. Lipids might be synthesized *de novo* in the clubroots, as the *proLTP1a*-GUS and *proLTP1b*-GUS showed increased GUS expression only at early infection stages. Defense-related JA and SA GUS marker genes did not markedly induce GUS expression in infected tissues, implying that *P. brassicae* avoids host defense responses.

Keywords: Arabidopsis, brassinosteroids, clubroot, *Plasmodiophora brassicae*.

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1 Introduction

Clubroot, caused by the obligate biotrophic protist *Plasmodiophora brassicae*, is a major problem in cultivated crops of *Brassica* species since the 13th century and possibly earlier (Dixon 2009). Worldwide, clubroot accounts for approximately 10% loss of production of *Brassica* oil and vegetable crops especially in temperate and moist climate zones. All species within the *Brassicaceae* family are potential hosts for *P. brassicae* including *Raphanus* and several weeds like *Arabidopsis thaliana* (Dixon 2009). Traditionally, clubroot has been managed by rotation with non-cruciferous crops. Nowadays, several other methods can also be applied to reduce the production losses including adding lime, calcium and boron in the soil (Donald et al. 2006). Still, these methods are not enough to stop the spread of the pathogen not least due to the long-lived resting spores that survive up to 20 years in infested soils.

P. brassicae is an obligate biotrophic protist and member of the Phytomyxea (parasites of plants, diatoms, brown algae and oomycetes) within the Rhizaria supergroup (Neuhauser et al. 2014). Phytomyxea are divided in two orders: Phagomyxida and Plasmodiophorida (parasites of plants and oomycetes). Plasmodiophorids include important plant parasites that cause significant diseases on *Brassica*, potato and grain crops (Neuhauser et al. 2014). *P. brassicae* has a typical obligate biotroph genome. The genome size is 24 kb and has a reduced amount of housekeeping genes (Schwelm et al. 2015). Still many questions remain regarding its disease process. It is anticipated that infection by *P. brassicae* involves two developmental stages (Figure 1). First, primary zoospores germinate from resting spores in the soil. Germination is triggered by root exudates (Dixon 2006). After germination, the zoospores move via a water film to the root hairs. The zoospores attach to the root hairs and inject their protoplast. In the root hairs *P. brassicae* grows into small plasmodia. These plasmodia produce secondary zoospores, which are released back into the soil and infect the root cortex. Within the cortex, larger plasmodia develop. This state is accompanied with hypertrophy and hyperplasia of the host cells leading to the clubbing phenotype. The development of clubs on the roots affects water and nutrient uptake in the plant and leads to wilting, stunting and premature ripening of the aboveground organs (Hwang et al. 2012). In the galls, resting spores are formed. These spores are released back into the soil when the plant decays after 5 weeks and start another infection cycle (Kageyama & Asano 2009).

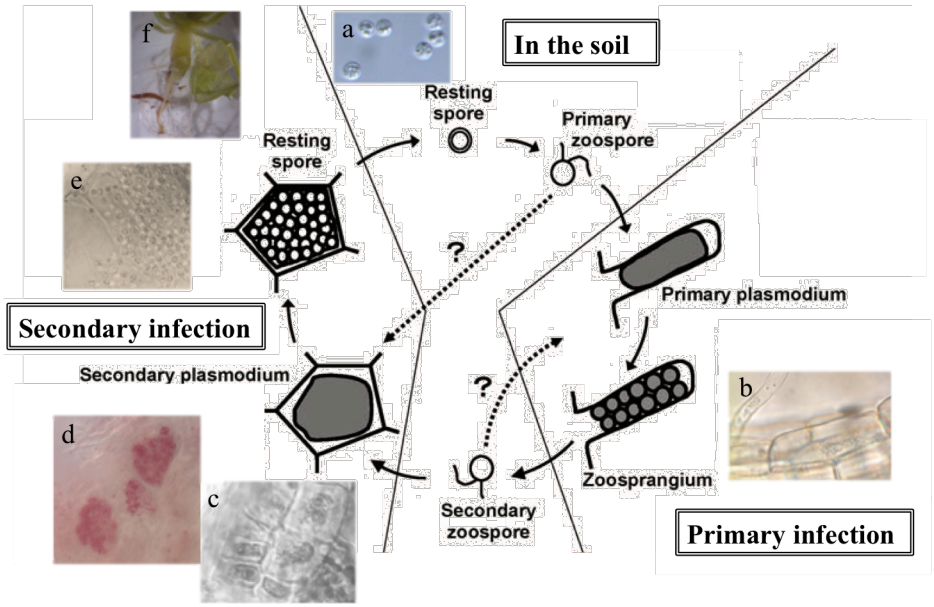

***Figure** 1*. Life cycle of *Plasmodiophora brassicae*. **a**. Resting spores, **b**. Root hair with cortical cell infected, **c**. Secondary plasmodia, **d** Fat red stained infected root cells, **e**. Resting spores inside cell, and **f.** Clubroot development on *A. thaliana* (Col-0). The life cycle is modified after Kageyama & Asano 2009.

During infection, *P. brassicae* alters the gene expression of the infected host (Siemens et al. 2006; Agarwal et al. 2011; Ludwig-Müller 2014; Schuller et al. 2014). Already in early infection stages many genes are differentially expressed in infected *A. thaliana* including genes involved in pathogen recognition and signal transduction (Siemens et al. 2006; Agarwal et al. 2011). Genes involved in oxidative burst and stress are down-regulated already 4 days post inoculation (Agarwal et al. 2011). After 2 weeks, more parts of the plant are infected while in the first week *P. brassicae* is mainly present in the root hairs. Signal transduction-related genes are higher expressed in later stages of the infection (Agarwal et al. 2011) as well as genes associated with reserve accumulation such as an increase in lipid starch proteins and starch synthesis (Siemens et al. 2006). *P. brassicae* accumulates fatty acids in developing spores. Interestingly, no fatty acid synthase is present in the genome of *P. brassicae*, thus the pathogen might be dependent on lipids from the host (Schwelm et al. 2015). Siemens et al. (2006) found that *LTP1* (LIPID TRANSPORT PROTEIN1) is up-regulated during infection, which support that hypothesis.

Hormones stimulate growth and cell division in root tissue and play an essential role during disease development (Devos et al. 2006; reviewed by Ludwig-Müller 2014). Cytokinin genes are for example down-regulated during infection with *P. brassicae* while overexpressing lines show higher resistance (Siemens et al. 2006). Jasmonic acid (JA) marker genes are differentially expressed during clubroot development (Siemens et al. 2006; Agarwal et al. 2011; Schuller et al. 2014). JA is known to play an important role in plant defense against necrotrophic pathogens. It was shown that JA biosynthesis genes are up-regulated during infection, for example *LOX4* (LIPOXYGENASE4) (Agarwal et al. 2011), while the signaling components *JAR1* (JA AMIDO SYNTHETASE1) and *COI1* (CORONATINE-INSENSITIVE1) are down-regulated (Schuller et al. 2014). In contrast, salicylic acid (SA) related genes were shown to be mainly down-regulated during infection (Agarwal et al. 2011). SA is a hormone that is involved in plant defense against various biotrophic pathogens. JA and SA are mutually antagonistic (reviewed by Kunkel & Brooks 2002). An increase of JA leads to resistance for a necrotroph

while SA increases susceptibility and vice versa for a biotroph (reviewed by Glazebrook 2005). SA induces pathogenesis-related proteins (PRs) which have antimicrobial activity (van Loon et al. 2006). *PR1* is down-regulated in infected roots (Siemens et al. 2006). *PR2* encodes β-glucanase, a gene that catalyzes callose turnover in the cell (Oide et al. 2013). Callose is considered as an important factor to decrease penetration of the cells during infection (Hückelhoven 2007). *PR2* activates the SA defense-signaling pathway. *PR2* might play a role in the balance between callose and the SA-dependent defense response at the infected site (Oide et al. 2013).

Brassinosteroids (BRs) might have an important role during disease development in for example *Xantomonas* (Jones 2001). BRs are growth promoting steroid hormones with structural similarity to cholesterol-derived animal steroid hormones (Clouse 2011). They are active in several complex metabolic pathways promoting cell division, cell expansion and differentiation. Developing plant tissue contains higher levels of BR than mature tissue and plant mutants with defects in BR biosynthesis or signaling have a dwarf phenotype. During *P. brassicae* infection, expression of BR genes is higher compared to non-infected plants (Siemens et al. 2006; Schuller et al. 2014). BR in the root might stimulate the cell division and elongation during gall development. Mutant plants with BR biosynthesis inhibitor showed reduced gall formation (Schuller et al. 2014). Furthermore, the *P. brassicae* genome contains an effector candidate for which the predicted protein has a structure similar to BR receptors (A. Schwelm, personal communication). BRs are synthesized from campesterol to brassinolide (BL) (Figure 2). *A. thaliana* mutants of the BR biosynthesis gene *DET2* (DE-ETIOLATED 2) are not able to produce BL due to a defect in the 5α-steroid reductase. This leads to increased amounts of campesterol in the plant (Clouse 2011) and resistance to *P. brassicae* (Siemens et al. 2002). BL is recognized by the *BRI1* (BRASSINOSTEROID INSENSITIVE1) receptor in the membrane. *BRI1* is a leucine-rich-repeat receptor-like kinase (LRR-RLK). When activated by BL, *BAK1* (BRI1-ASSOCIATED RECEPTOR KINASE1 or SERK3), a member of the LRR-RLK SERK subfamily, binds to *BRI1*. This leads to downstream signaling via *BSK1* (BR-SIGNALING KINASE1) and *BES1*/*BZR1* transcription factors and biosynthesis of BL (Figure 2) (Clouse 2011). *BAK1* has multiple functions including BR signaling and plant defense. It has been proposed that *BAK1* has an independent function to control cell death during infection (Kim et al. 2013). Controlled cell death is important for resistance against biotrophs. *BAK1* is important for MAMP recognition and downstream signaling (Domínguez-Ferreras et al. 2015). Furthermore, *BAK1* interacts with *FLS2* (FLAGELLIN SENSITIVE2). *FLS2* is known to have a function in plant defense against the *FLS* from bacterial pathogen *Pseudomonas syringae*, but the defense mechanism against different pathogens differ. *FLS2* has been characterized as the pattern-recognition receptor (PRR) for flagellin and elongation factor Tu (EF-Tu) (Chinchilla et al. 2007). The *FLS2*/*BAK1* complex illustrates that *BAK1* is a cofactor during initial responses of plant-triggered immunity.

The model plant *A. thaliana* can be a useful tool to study host pathogen interactions due to the availability of a vast number of mutant and reporter genotypes. Several plant mutants were tested including endochitinase, ubiquitin, fer1, gcp2, al7 and tre1 mutants. These mutants might have features that are affected by *P. brassicae* during infection. *P. brassicae* spores contain trehalose and chitin. This pathogen is able to produce trehalose and chitinases itself (Brodmann et al. 2002; Schwelm et al. 2015). Furthermore, *P. brassicae* might interfere with the ubiquitination system of the host (Schwelm et al. 2015).

To study the interaction between *P. brassicae – A. thaliana* a consistent infection system needs to be established. To minimize the influences of other external factors (organic matter, micro-organisms, etc.) a sterile system would be more favorable compared to the soil. However, *P. brassicae* is an obligate biotroph and needs to be extracted from the plant, which is far from sterile. Callus culture could be established (Asano et al. 1999; Bulman et al. 2011) but a more natural system is needed to understand the effect of *P. brassicae* in agricultural fields. Three different plant cultivation systems were tested to study the infection progress of *P. brassicae*: soil, sand and liquid culture. The most suitable infection system was used to test mutant and reporter strains.

The aim of this thesis was to:

- Establish a suitable infection system for the *A. thaliana – P. brassicae* interaction.
- Use the best infection system to study:
    - transgenic *A. thaliana* genotypes harboring GUS constructs driven by gene promoters involved in defense, hormones and lipid metabolism.
    - selected *A. thaliana* T-DNA mutants for their response to *P. brassicae*.
- Transform *A. thaliana* Col-0 with a *P. brassicae* effector candidate.

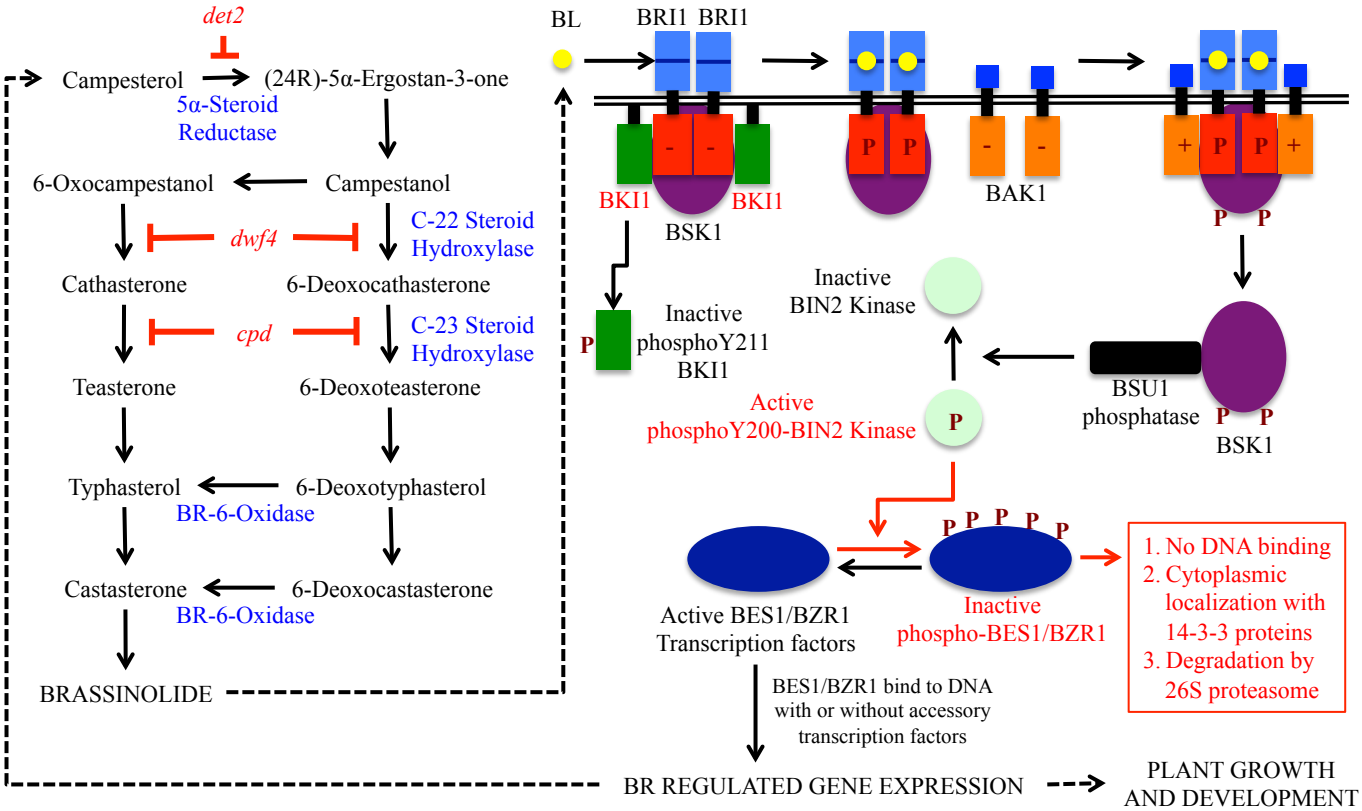

Figure 2. Brassinosteroid biosynthesis and signaling model (modified after Clouse 2011).

2 Material and methods

2.1 Plant and pathogen material

Seeds of *A. thaliana* Ler-0 and Col-0 and the mutants in Table 1 were used for the experiments. All mutants had a Col-0 background except of *fer1* that had a Ler-0 background. The *A. thaliana* seeds were surface sterilized by incubating in 10% chlorine for 10 min followed by incubation in 70% ethanol for maximal 1 min. Afterwards, the seeds were washed several times with sterile distilled water with 10 min between each washing step. The sterile seeds were plated on MS-agar and grown in an 18h light period at 22°C and 20°C in the dark. The heterozygous T-DNA mutants were grown on MS-agar plates containing 100 mM kanamycin. For all experiments, at least 20 infected and 20 non-infected plants were used.

P. brassicae isolate e3 (Fähling et al. 2004) was used to infect the different plant genotypes. Spores were extracted from fresh Chinese cabbage clubs (Figure 3). The clubs were washed to remove the soil particles. The galls were mixed in water to get a homogeneous plant suspension using a household mixer. The suspension was filtered through several layers miracloth to remove most plant debris. Afterwards, the suspension was centrifuged for 10 min at 1500 g. The supernatant was discarded and the layer above the white starch layer was resuspended and transferred into a new tube and centrifuged again for 10 min at 1500 g. The supernatant was discarded and the spores were surface sterilized with 70% ethanol and centrifuged again directly. The ethanol was removed and spores were washed with 15% household bleach. After centrifugation and removing the bleach the spores were resolved in sterile water. The spores were purified using 16% 32% ficoll centrifugation for 40 min at 450g (Asano et al. 1999). The spore solution was added on top of both ficol layers. After centrifugation, the spores between the 16% - 32% ficoll layers were carefully collected using a syringe. The spores were pelleted by centrifugation (1 min, 1000 rpm) and the spores were incubated for 20 min with 2% chloramine-T. After centrifugation the supernatant was removed and the spores were washed again with distilled water. The amount of spores was counted with a hemocytometer and divided in aliquots and stored at -20°C until further use.

Figure 3. Fresh cabbage clubs used for extracting *P. brassicae* spores.

Table 1. *Mutants of A. thaliana that were used in this study*

Name / gene ID	Proposed gene function	Ref.
***A. thaliana* T-DNA mutants**		
fer1	Transcription factor in response to auxin	(Tian et al. 2005)
SALK_099504: * mutation between At2g43610 and At2g43620	Both are endochitinases with pathogen response and biogenesis of cell wall as proposed functions	(Passarinho & de Vries 2002)
SALK_143296: * At4g01037	Ubiquitin carboxyl-terminal hydrolase superfamily protein	(Alonso et al. 2003; Kroeger et al. 2009)
SALK_032503C: *al7*, *alfin-like 7*, *atal7*	Salt stress induced gene, negative role in salt tolerance	(Alonso et al. 2003; Song et al. 2013)
SALK_151478C: *atbcp2*, *gcp2*, *tubg2*	γ-tubulin complex protein	(Alonso et al. 2003; Seltzer et al. 2007)
SALK_081943C: At1g02360	Endochitinase: biogenesis of cell wall	(Passarinho & de Vries 2002)
SALK_147973C: *attre1*, *tre1*, *trehalase1*	Induces trehalase activity	(Brodmann et al. 2002)
Transgenic promoter - GUS genotypes		
proBRI1-GUS	Recognition of BR	(Clouse et al. 1996)
proBAK1-GUS	Signaling in BR	(Li et al. 2002)
proDET2-GUS	Biosynthesis of BR; 5a-steroid reductase	(Li et al. 1997)
proPR1-GUS	Pathogenesis related 1; SA responsive marker gene	(Segarra et al. 2013; van Loon et al. 2006)
proPR2-GUS	Pathogenesis related 2; B-1,2-glucanase, modulates callose and SA dependent defense responses	(Oide et al. 2013)
proLOX2-GUS	Lypoxygenase 2; JA marker gene upstream in the JA biosynthesis	(Bell et al. 1995)
proVSP2-GUS	Vegetative storage protein 2; JA marker gene	(Utsugi et al. 1998)
proLTP1a-GUS	Lipid transporter protein 1a	(Segura et al. 1993; García-Olmedo et al. 1995)
proLTP1b-GUS	Lipid transporter protein 1b	
proPDF1.2-GUS	Ethylene and JA responsive plant defensing factor 1.2	(Lu et al. 2013)
Transgenic promoter - GFP genotype		
pro35S:PIP2-GFP	GFP labeled plant membrane	(Moschou et al. 2013)

* These T-DNA mutants were heterozygous, all other used *A. thaliana* mutants were homozygous.

2.2 Plant growing conditions

2.2.1 Soil

A. thaliana seedlings grown for approximately one and a half week on MS agar (0.8% bacto agar, 1% sugar and 4.4 g MS per L, pH 5.7) were transferred to the soil and grown at 22°C and 6 hours of daylight. After two days in the soil, the plants were infected with 2 ml *P. brassicae* spore solution (1 x 10^6 spores/ml H_2O) (Kobelt et al. 2000). 2 ml H_2O was added to the control plants. The plants were watered three times a week and were grown for another four weeks under the same conditions (Figure 4). Three to four weeks post inoculation (21 – 28 dpi) with *P. brassicae*, the plants were analyzed to determine if clubs had developed. The GUS reporter genotypes were analyzed more often (time series of two times a week post inoculation) to identify if GUS expression was different between the different infection stages.

***Figure** 4.* Soil system (plants were 5 weeks old in the left and top right and 3 weeks in the bottom right picture).

2.2.2 Liquid system

Pipet boxes with liquid medium were used to make a sterile environment for the plants to grow (Figure 5). 1 ml pipet tips were cut to 1 cm in length. 16 pipet tips were put in each box. The other holes were covered with aluminum foil to prevent light below the tips. After autoclaving, the pipet tips were filled with MS agar. On top of the agar 1 germinated Col-0 seedling from MS-plates was added. The boxes were incubated at an 18h light period at 22°C and 20°C in the dark. When the plants were approximately 1.5 weeks old, the roots started to grow through the agar. The boxes were filled with liquid ¼ MS medium (1.1 g MS per L, pH 5.7) to the basis of the tips. When most roots reached the liquid (approximately when the plants were two weeks old), the boxes were inoculated by adding 200 µl *P. brassicae* spore solution (1 x 10^7 spores/ml H_2O) to the roots. The medium was refreshed once a week. The plants were analyzed approximately 21 dpi.

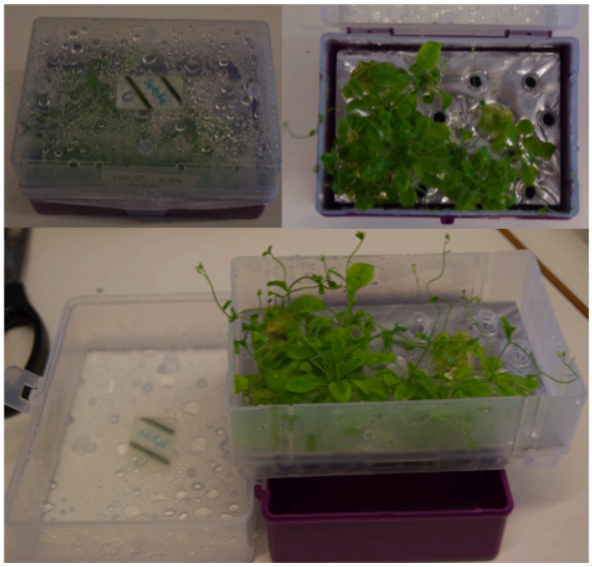

Figure 5. Liquid growing system.

2.2.3 Sand box system

The pipet box system was modified to a sand system to prevent a different phenotype of the roots between the soil and the liquid system (Figure 6). In the sand system, the pipet boxes were filled with approximately 3 cm of a mixture of sand and agra-perlite soaked in ¼ MS medium, pH 5-5.5. After autoclaving the boxes, 8-10 small plant seedlings (approximately one week old) were placed on top of the sand mixture. When the plants had 4 leaves (approximately 1.5 weeks old), each plant was inoculated with 200 µl *P. brassicae* spore solution (1 x 10^7 spores/ml H_2O). At 7 dpi, the lid was removed to prevent water stressed plants due to the high humidity in the boxes. Twice a week, the boxes were watered with ¼ MS medium, pH 5-5.5. The pH was kept low to stimulate infection (Luo et al. 2013). Three to four weeks after infection, the plants were analyzed to determine if clubs developed.

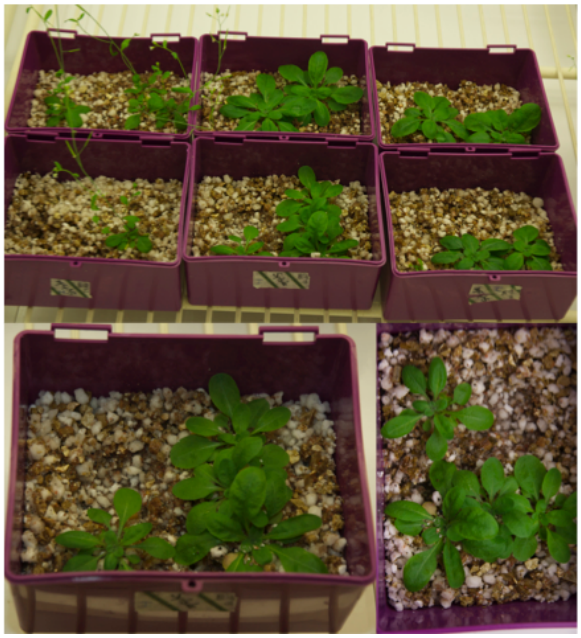

Figure 6. Sand box growing system.

2.3 Staining procedures

2.3.1 GUS staining

The *A. thaliana* promoter-GUS genotypes (Table 1) were analyzed twice a week after infection up to 28 dpi. The plants were grown in the soil as described in 2.2.1. The roots were washed and stained with X-Gluc solution (830 µl H_2O, 100 µl 1M NaPO4 (pH 7), 20 µl 0.5M EDTA, 10 µl 10% Triton X-100, 20 µl 50mM K3Fe(CN)6 and 20 µl 0.1M X-Gluc (dissolved in N,N-DMF) per 1 ml) to detect the GUS activity in the roots (Jefferson 1987). The tissue was immerged in the solution and briefly vacuum infiltrated. The tissue was incubated overnight at 37°C. *proPR2-*, *proVSP2-*, *proLOX2-* and *proPDF1.2*-GUS lines were only incubated for 2 hours due to the high expression of the genes. After incubation, the roots were washed with 70% ethanol several times until the tissue cleared. Between each EtOH change, the plants were incubated on a shaker for at least 3-4 hours. The samples were kept in 50% EtOH at 4°C. Cross-sections of the infected roots from BR GUS-reporter strains were made from 18 dpi with a razor blade. Analysis of the whole roots and cross sections of the roots was done using light microscopy (Leica MZ6).

2.3.2 Lipid staining

Lipid staining of Col-0 plants was done to determine the presence of lipids in infected roots. Roots of Col-0 were harvested when clubs were visible to be sure that the roots were infected. Fat red stain was added to whole roots and cross-sections of the roots. The stain was incubated for 15 min at room temperature. Uninfected Col-0 plants were used as control. Furthermore, Nile red and Toluidine blue was used to stain cross-sections of clubs from cabbage.

2.4 Analysis of *A. thaliana* deficiency mutants

The *A. thaliana* mutants (Table 1) and Col-0 and L*er*-0 were grown in soil as described in 2.2.1. After 28 dpi, 20 infected and 20 non-infected plant roots were harvested. Pictures of the roots were made and the weight of the roots was measured. The roots were frozen in liquid nitrogen in samples of 5 roots and stored at -70°C before further analysis. DNA was extracted from the infected roots using the Qiagen DNeasy Plant Mini Kit. The concentration of the DNA was measured with nano drop. PCR was used to measure the amount of *P. brassicae* DNA with *P. brassicae* specific primers (two sets of primers: PbF3 and PbR3 (Chai et al. 2015) and Pb4-1 with PbITS6 (Sundelin et al. 2010)). AtrtFw and AtrtRw primers were used to measure *A. thalian*a DNA (Gachon & Saindrenan 2005).

2.5 *pro35S:PIP2*-GFP

The plants were grown in the soil. Four weeks after infection, the plants developed clubs. The roots were harvested and analyzed with a confocal microscope (LSM Zeiss 780) to identify if plant membranes surrounded the infection structures in the root and if this method could be used to observe the disease progress.

2.6 Agrobacterium-transformation of *A. thaliana* using *P. brassicae* genes

A. thaliana Col-0 was transformed with a *P. brassicae* effector candidate for which the protein had a predicted structure similar to BR receptors. This material could be used to test if expression of this gene shows abnormalities in BR gene expression. First, the effector was amplified from 2 *P. brassicae* isolates Pb e3 and Pb P2 DNA. Primers were designed and ordered by Invitrogen™. Two different forward primers were designed to amplify the effector both with and without its signal peptide. PCR was used to amplify the gene using *P. brassicae* genomic DNA. Cloning of the DNA sequence was performed using the CloneJET PCR cloning kit (Blunt-End ligation protocol, Life technologies). The plasmids were extracted after cloning using the pJET Plasmid Purification Kit (Life Technologies). The product lengths were checked with *Bgl*II restriction enzyme (Life Technologies) and the plasmids were sent away for Sanger sequencing (Macrogen).

A PCR reaction (2 min 95°C, 25x (30 sec 95°C, 30 sec 58°C and 1 min 72°C), 5 min 72°C) with DreamTaq DNA Polymerase was used to amplify the sequence from the plasmids and to add a 3-A tail. The PCR products were used for GATEWAY™ cloning in TOP10 *E. coli* cells using pCR8 as TOPO cloning vector and both pGWB5 (Nakagawa 2002) and pGWB502 (accession number AB294469) as GATEWAY™ destination vectors. The length of the inserts in the pGWB vectors were checked with *Hin*dIII and *Sac*I restriction enzymes (Life Technologies) and the plasmids were sent away for sequencing (Macrogen). The plasmids with the correct sequences were transferred into Agrobacterium cells with T2 helper plasmid according to the protocol. 200 ml of Agrobacterium suspension containing the plasmids was spinned at 4000 rpm for 8 min. The pellet was dissolved in 40 ml IF medium (1/2 MS and 5% sucrose) with 16 µl Silwet-77 solution. The floral dip method was used to transform the plants (Clough & Bent 1998). Dipping the flowers was repeated after 7 days. The seeds were harvested.

3 Results

3.1 Growing systems

Col-0 plants frequently developed clubs when grown on soil after 21-28 dpi (Figure 7). First swollen roots were found 18 dpi and galls from 21 dpi. After four weeks, the plants started wilting.

In liquid medium no clubs or swollen roots were observed 21 dpi. Plants showed altered root morphology probably due to water stress. After 21 dpi fungi started to grow in the medium and it was not possible to grow the plants for a longer period. The medium could not be kept sterile, as the purified *P. brassicae* spore inoculum is never absolute pure and contains small amount of fungal spores.

In the sand boxes Col-0 root morphology was more normal. The root hairs were longer and a higher amount of roots could be harvested compared to the soil, because it was easier to remove the sand from the roots without breaking them. However, clubs developed only in one plant (in *proPDF1.2*-GUS). Microscopic analyses did not conclusively show an infection of the root hairs deriving from the sand system.

As infections were only consistently achieved with plants grown in soil, this system was used in all following studies.

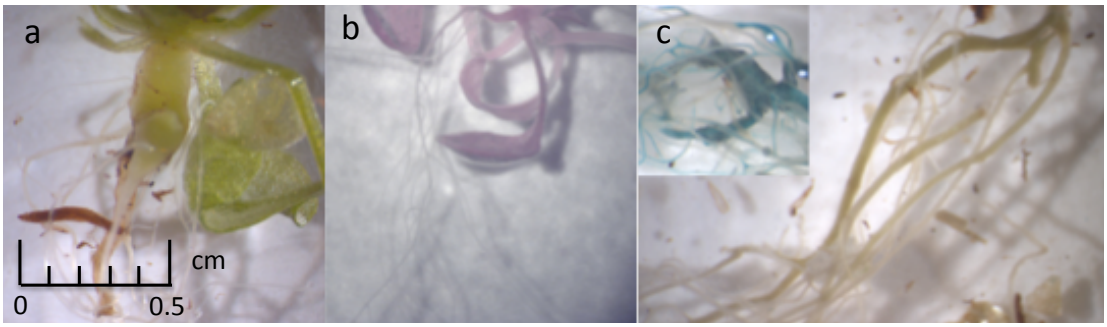

Figure 7. Infected Col-0 plants from the different growing conditions**: a.** Soil (28 dpi), **b**. Liquid (21 dpi) and **c**. Sand system (28 dpi). Small picture in c is a stained club from *proPDF1.2*-GUS.

3.2 GUS expression in brassinosteroid promotor genotypes

Expression of BR related genes were investigated using *proBAK1*-GUS, *proBRI1*-GUS and *proDET2*-GUS reporter lines grown in soil. Twice a week, plants were analyzed to determine the GUS expression in the roots. In control plants, no GUS expression was observed. In *proBAK1*-GUS lines GUS was highly expressed in infected roots and clubs from 14 dpi (Figure 8). The same expression pattern was found for *proBRI1*-GUS genotypes (Figure 9). GUS was exclusively active in the root tips of control *proDET2*-GUS plants. In infected roots, GUS was additionally expressed from 10 dpi in clubs and swollen roots (Figure 10).

Cross-sections of *proBAK1*-GUS and *proBRI1*-GUS lines showed GUS expression in the whole club. GUS was mainly expressed near the root cortex in the clubs of *proDET2*-GUS materials (Figure S1).

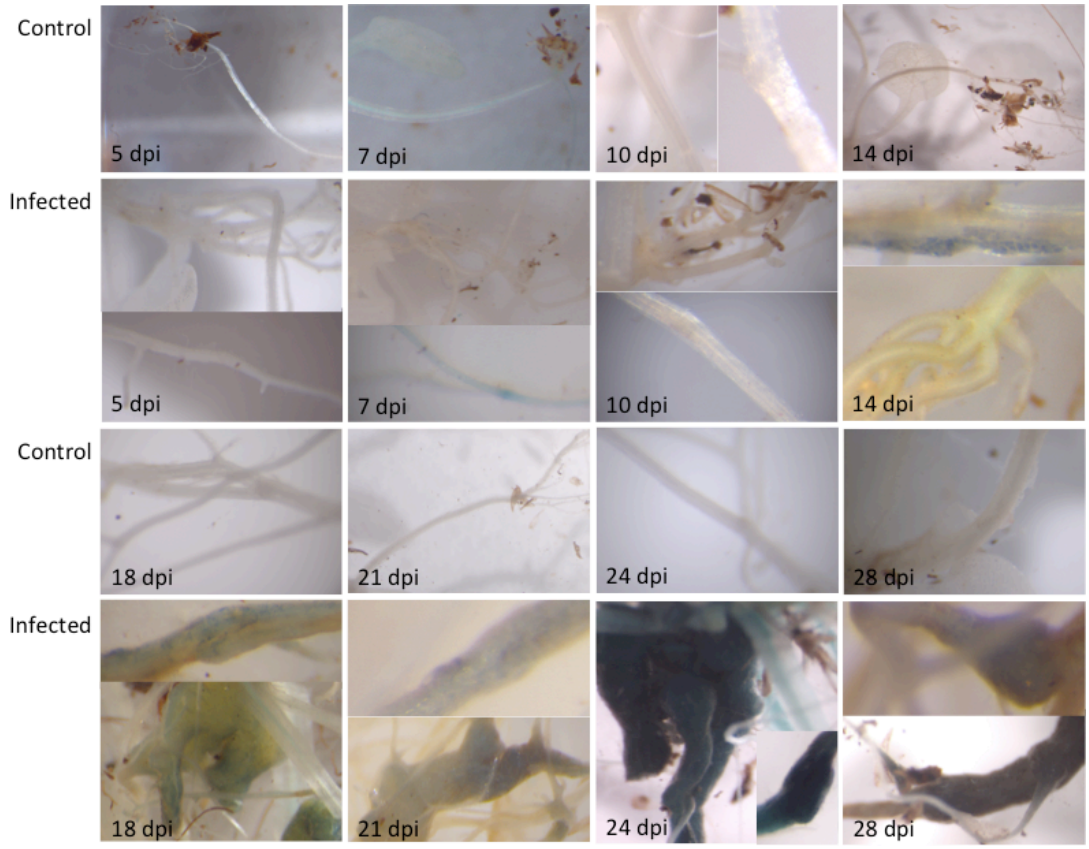

***Figure** 8.* Overview of GUS stained *proBAK1*-GUS materials.

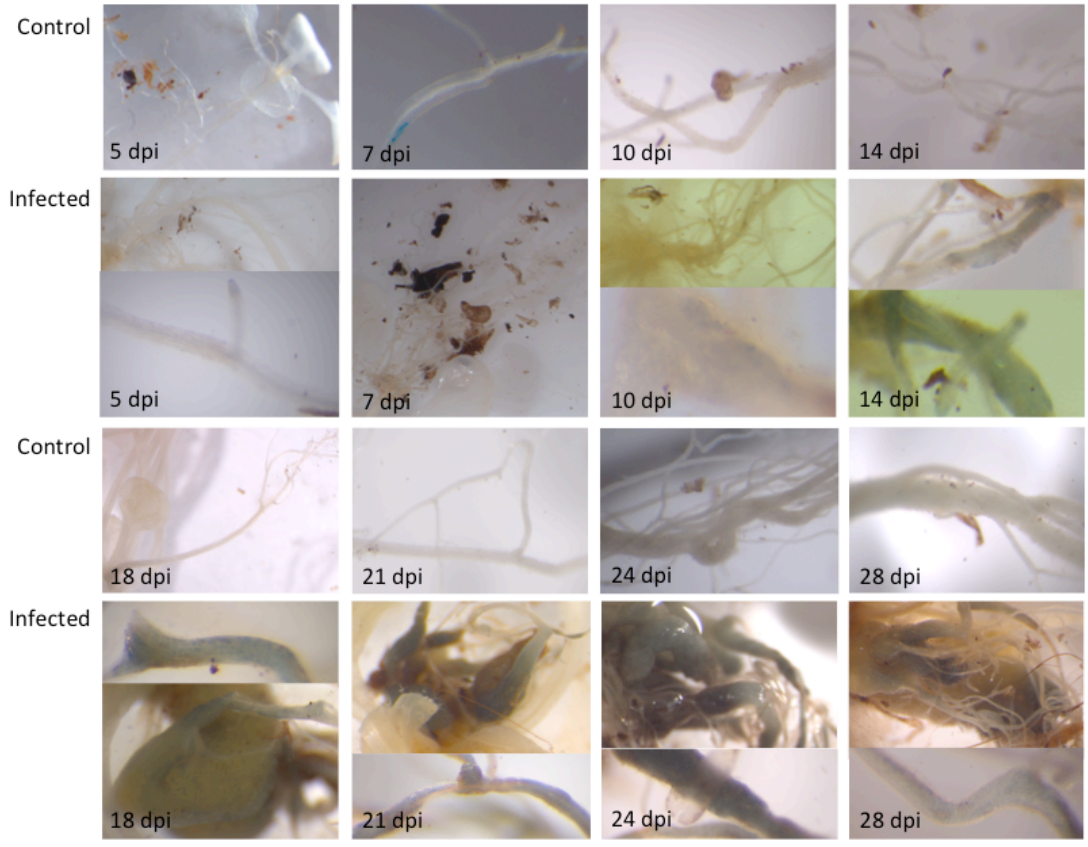

Figure 9. Overview of GUS stained *proBRI1*-GUS materials.

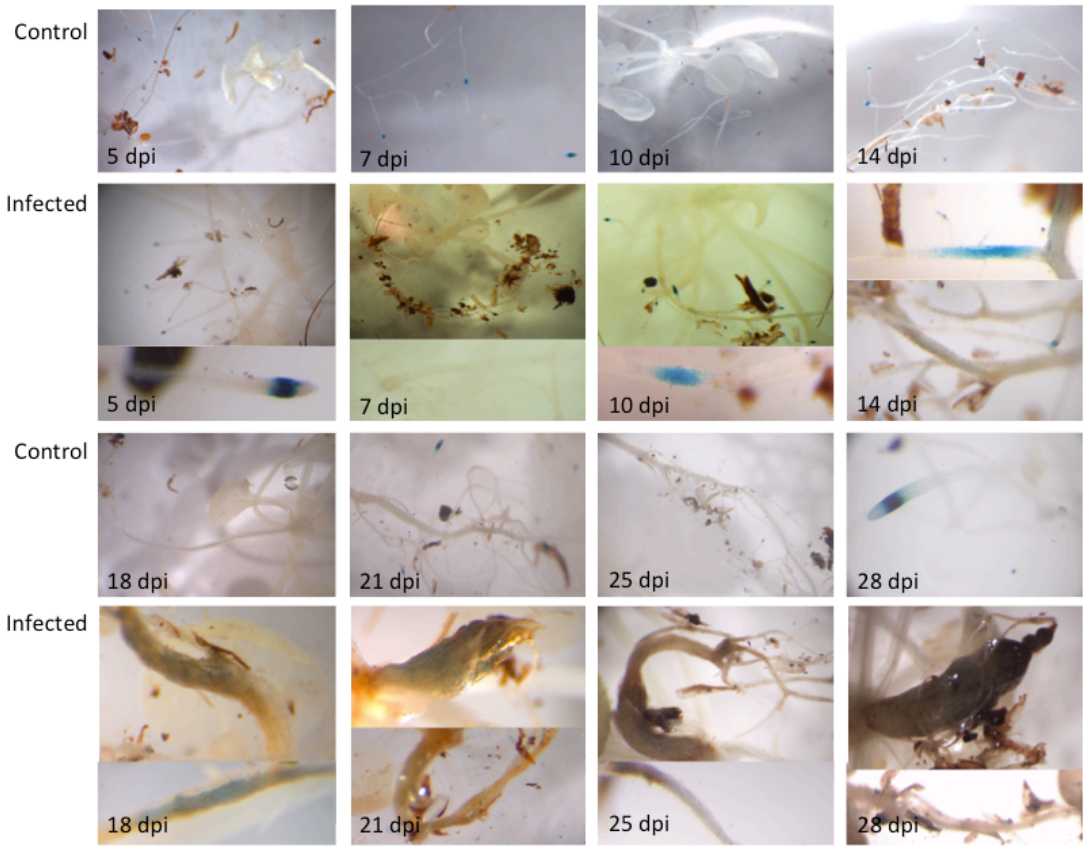

Figure 10. Overview of GUS stained *proDET2*-GUS materials.

3.3 Lipids

3.3.1 Lipid staining

Galls and root tissue of Col-0 and Chinese cabbage (*Brassica rapa*) infected with *P. brassicae* contained a high number of lipid bodies (Figure 11 & 12), which were absent in roots of control plants.

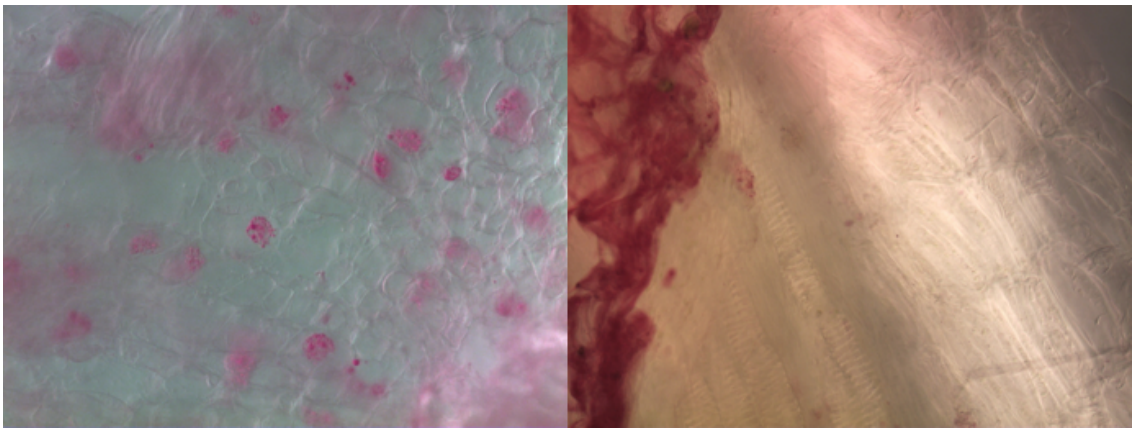

Figure 11. Infected (left) and non-infected (right) Col-0 root tissue with fat red staining.

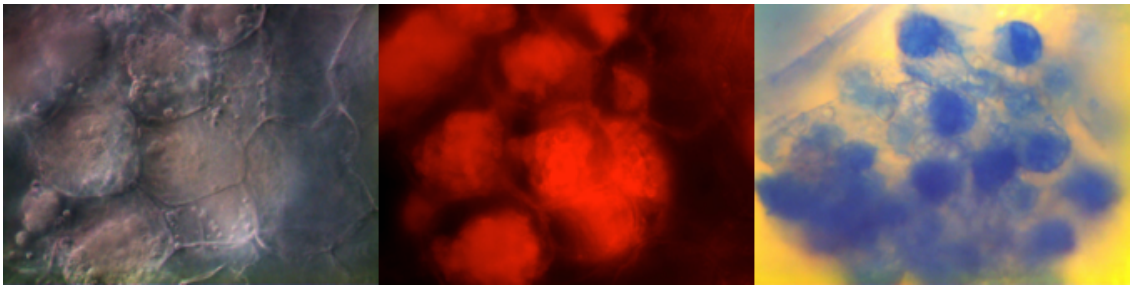

Figure 12. Club of Chinese cabbage (400x) (left) stained with nile red (middle) and toluidine blue (right).

3.3.2 GUS expression in *proLTP1a*- and *proLTP1b*-GUS lines

proLTP1a- and *proLTP1b*-GUS reporter genotypes were investigated to test if GUS expression for both lipid transporter proteins was higher in infected root tissue. Both GUS reporter lines showed GUS expression in primordia of developing side roots (Figure 13 & Figure 14) in uninfected and infected roots. Occasionally, GUS expression was seen in other parts of the roots for both proteins exclusively in infected roots after 21 dpi. In developed clubroots, no increased GUS expression was observed.

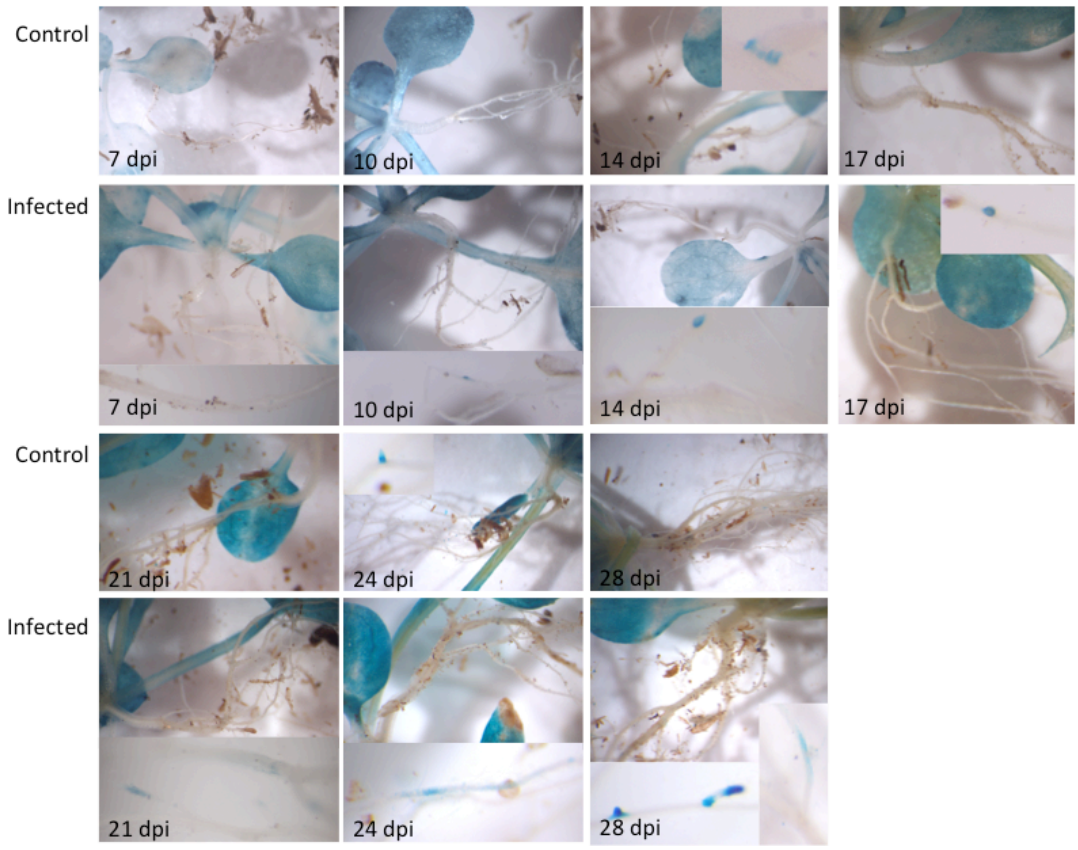

Figure 13. Overview of GUS stained *proLTP1a*-GUS materials.

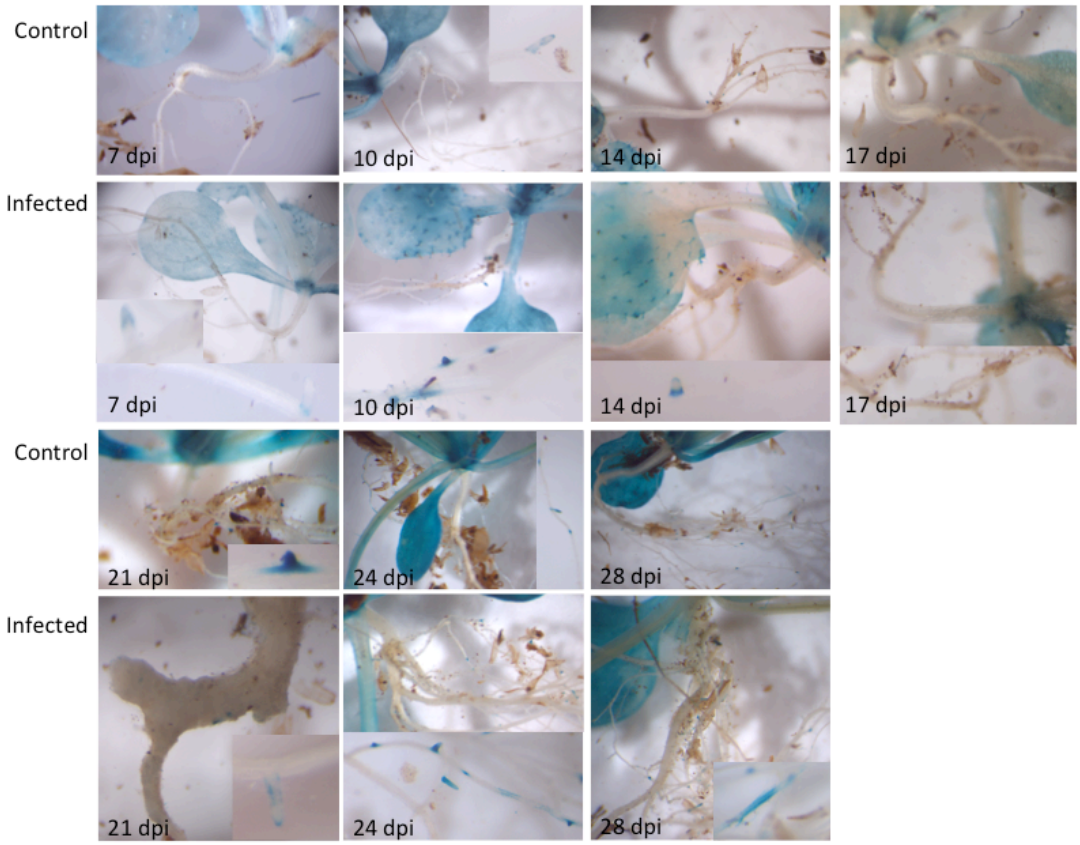

Figure 14. Overview of GUS stained *proLTP1b*-GUS materials.

3.4 GUS expression in *proPR1*- and *proPR2*-GUS lines

For both *proPR1*- and *proPR2*-GUS genotypes, no differences were found in GUS expression between non-infected and infected plants. Only small leaves of larger plants and small root patches showed GUS expression for *proPR1*-GUS (Figure 15). GUS was consistently expressed in the leaves and patches of the roots in both the infected and control plants of *proPR2*-GUS (Figure 16).

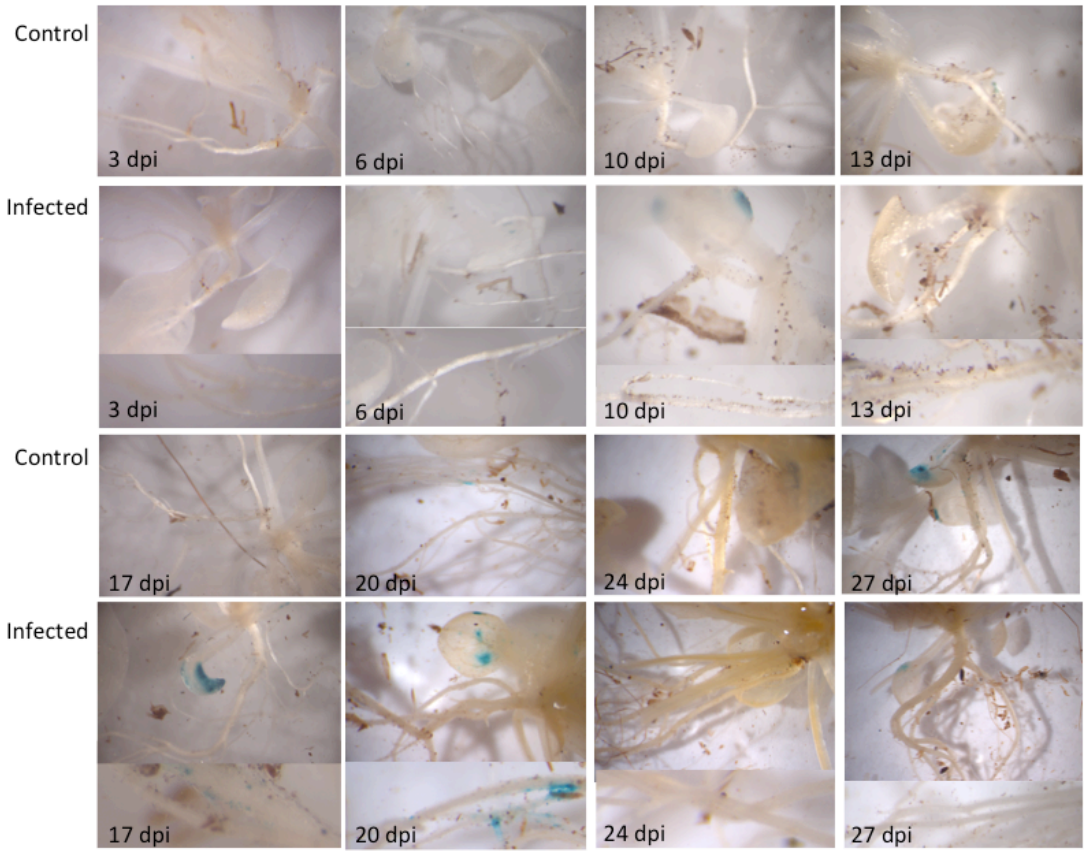

Figure 15. Overview of GUS stained *proPR1*-GUS materials.

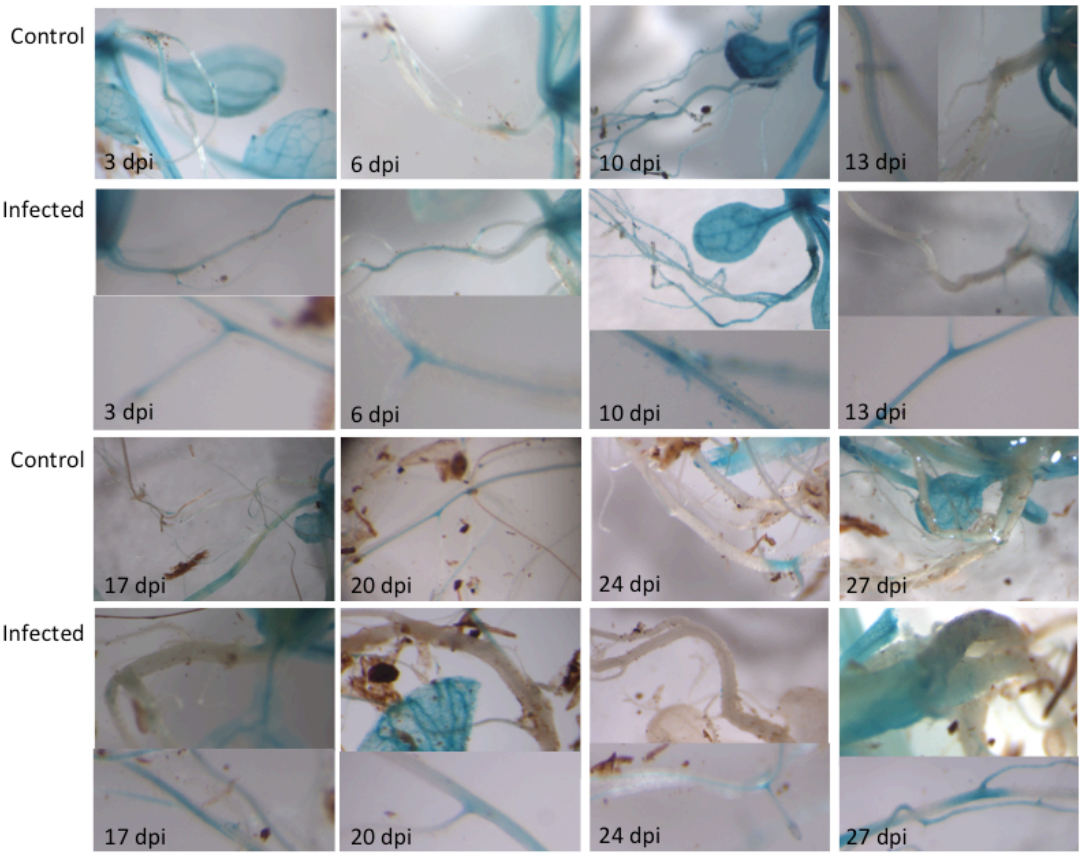

Figure 16. Overview of GUS stained *proPR2*-GUS materials.

3.5 Jasmonic acid marker genes

3.5.1 *proPDF1.2*-GUS lines

GUS reporter plants of the JA/ethylene responsive *PDF1.2* showed no differences between control and infected plants. GUS was expressed in patches in the roots for both infected and control plants and additionally in clubroots (Figure 17).

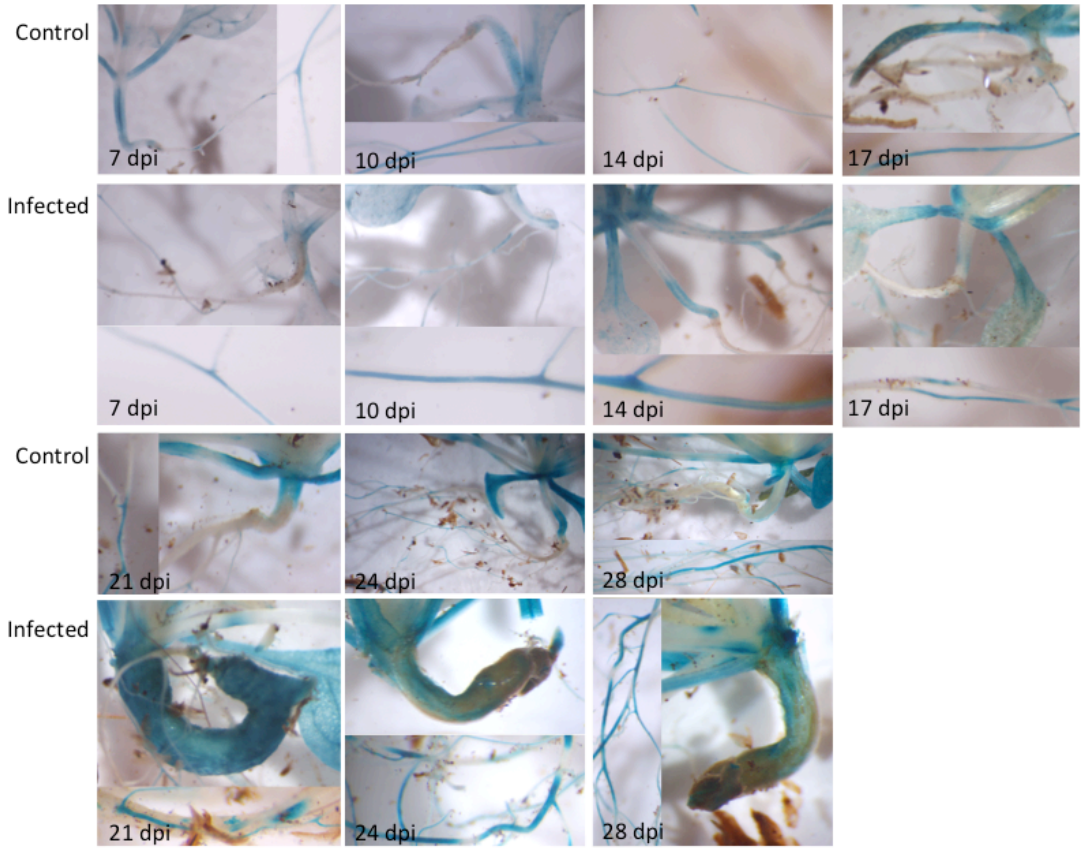

Figure 17. Overview of GUS stained *proPDF1.2*-GUS materials.

3.5.2 *proLOX2*- and *proVSP2*-GUS lines

Similar to *proPDF1.2*-GUS, GUS expression was not markedly up- or down-regulated during infection for both JA marker genes *LOX2* and *VSP2*. GUS expression was only observed in some parts of infected roots from 14 dpi in *proLOX2*-GUS (Figure 18) and GUS expression did not differ between control and infected plants in *proVSP2*-GUS (Figure 19).

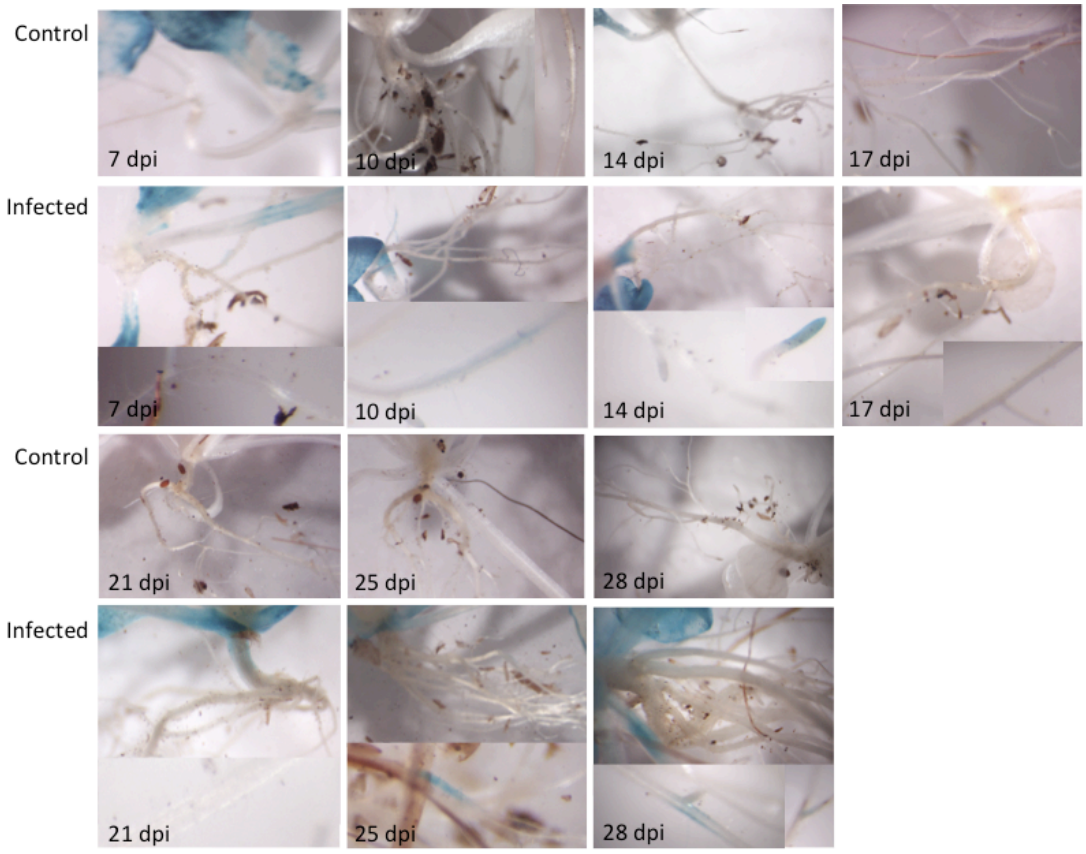

Figure 18. Overview of GUS stained *proLOX2*-GUS materials.

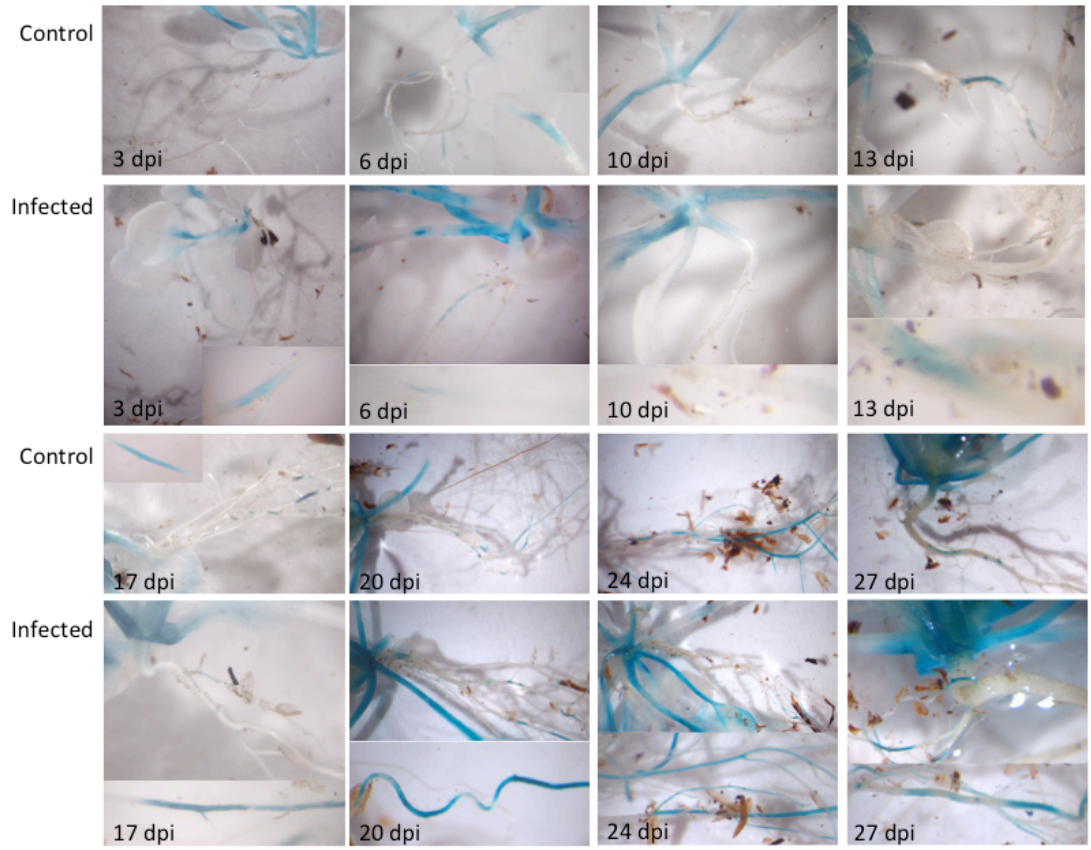

Figure 19. Overview of GUS stained *proVSP2*-GUS materials.

3.6 T-DNA mutants

Seven different mutants (Table 1) and two wild type *A. thaliana* plants were grown in the soil. All mutant plants developed clubs (Figure 20). SALK_081943 endochitinase mutant showed only small thickening of the roots during infection. However, from all 20 analyzed infected plants only a few developed clubs even in the Col-0 wild type plants. For most plants, the root weight was higher in infected plants compared to control plants (Table 2).

Table 2. *Weight (in gram) of harvested plant roots from the wild type and deficiency mutants.*

	Infected	Control	ri/rc*
Col-0	0.034	0.042	0.810
L*er*-0	0.145	0.057	2.544
fer0	0.141	0.066	2.136
SALK_099504 **	0.110	0.057	1.930
SALK_143296 **	0.109	0.137	0.793
SALK_081943	0.137	0.175	0.778
al7	0.142	0.090	1.581
gcp2	0.141	0.098	1.431
tre1	0.078	0.054	1.448

** Ratio weight infected/control roots*

*** Both T-DNA mutants were heterozygous*

Figure 20. Infected T-DNA mutants and wild type plants 28 dpi.

DNA was extracted from roots to determine the amount of *P. brassicae*, but no pathogen DNA as well as *A. thaliana* DNA could be amplified by PCR (Figure 21). The DNA concentration was too low in all samples (ranging from 0.5 to 5.0 ng/µl) for the PCR. Due to the time limit, it was not possible to identify the problem in the PCR.

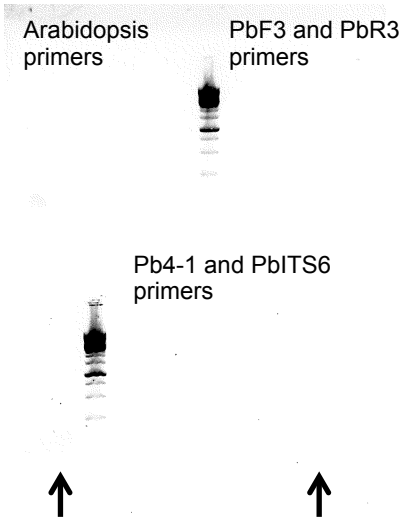

Figure 21. Agarose gel (with 1kb ladder) of six extracted *P. brassicae* DNA samples with the highest concentration (5 ng/µl) after PCR with the *P. brassicae* and *A. thaliana* primers. *P. brassicae* P2 DNA was used as positive control (arrows). No DNA could be amplified.

3.7 *pro35S:PIP2*-GFP

It was tried to monitor the infection progress with a GFP labeled plasma membrane mutant using confocal microscopy. A putative plasmodia surrounded by host plasma membrane was seen once (Figure 22). The time frame of this thesis did not allow optimizing the sample preparation to study the infection in more detail using confocal microscopy.

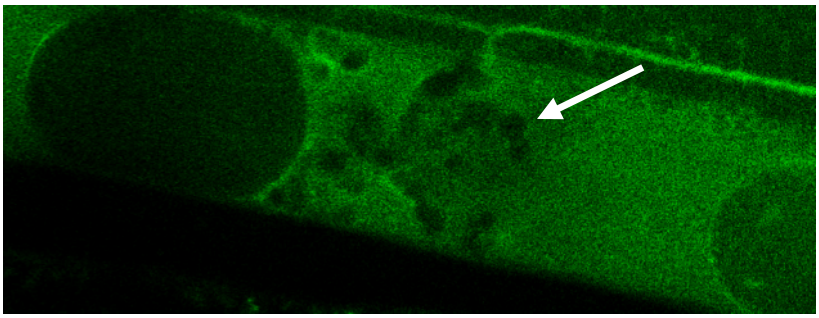

Figure 22. Confocal image of *pro35S:PIP2*-GFP at an infection site in the root. The white arrow indicates a possible infection structure (plasmodium).

3.8 Agrobacterium transformation of *A. thaliana* using *P. brassicae* genes

A. thaliana transformation was done with two different *P. brassicae* genes. Cloning of *E. coli* and Agrobacterium with the *P. brassicae* sequences of the effector candidate was successful. The pGWB plasmids did contain the right insert validated with sequencing and with the *Hin*dIII and *Sac*I restriction enzymes (Figure 23).

Due to the time limit within this thesis, it was not possible to analyze the phenotype and measure altered BR gene expression in the transformed plant.

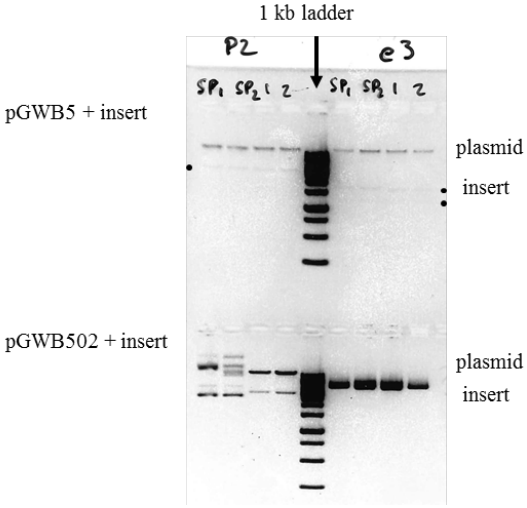

Figure 23. Agarose gel (with 1kb ladder shown in the middle) of pGWB5 and pGWB502 products (sp1 and sp2: with signal peptide in the insert; 1 and 2: without) for both P2 and e3 *P. brassicae* isolates after cutting the plasmid with *Hin*dIII and *Sac*I enzymes. The E3 isolate contains a *Hin*dIII cutting site inside the selected gene (showing 2 bands for the insert). The dots indicate weak bands.

4 Discussion

For the well-studied model plant *A. thaliana* are a vast number of mutant and reporter strains available. Therefore, *A. thaliana* can be a valuable tool to study the interaction between the obligate biotrophic protist *P. brassicae* in this host.

A soil-free system would be desirable to study the infection and host interaction of *P. brassicae* in *A. thaliana*. In the soil, external factors (including organic matter and micro-organisms) can influence the gene expression in the plant. Furthermore, the roots break easily in the soil when harvesting the plants. Infection in liquid cultures has been successfully reported from *B. oleracea* as shown by Luo et al. (2013). However, we could not establish *A. thaliana* plants in a liquid or sand system. Similar, Agarwal et al. (2009) could not establish the clubbing phenotype during *P. brassicae* infection of *A. thaliana* grown in a sand culture. Consistent infection occurred only when the plants were grown in the soil. Therefore, although difficult to remove and clean the roots, this was the system of choice to study the interaction between *P. brassicae* and *A. thaliana*.

When the mutant plants were tested, no visible differences of infection was found between the mutants and the wild type. But even in the soil, not all infected plants developed clubs, even grown in the same pot and infected with the same spore solution. Therefore it is difficult to make an assumption of infection level in the mutants compared to the wild type especially since the PCR did not work. The DNA concentration was too low to detect both *P. brassicae* and *A. thaliana* DNA. The primers for *P. brassicae* did not show the right product length in the positive control either. Within this thesis, the time limit did not allow troubleshooting. Mutant analysis might be a useful technique to determine visible differences between infected and non-infected plants, but Siemens et al. (2002) screened 71 mutants and found in only four lines a certain degree of tolerance against *P. brassicae*. These mutants were tested to develop a useful system to screen a larger set of mutant lines. Other methods such as gene expression analysis might be more useful determine the infection strategy of *P. brassicae*.

Several GUS-reporter lines, including genes involved in BR biosynthesis and signaling, lipid transport and the SA and JA pathway (Table 1), were studied to determine if *P. brassicae* affects the GUS expression in the reporter strains.

In the BR GUS reporter line, GUS expression was increased in *P. brassicae* infected tissue. A higher amount of BR might provide the pathogen with growing host tissue, which can be exploited for nutrients. We could show that promoters from signaling genes, *BRI1* and *BAK1*, as well as the biosynthesis gene *DET2* induced higher GUS expression in infected root tissue. This supports other studies which revealed a reduced infection and gall development in *det2* and *bri1-6* mutants (Siemens et al. 2002; Schuller et al. 2014). How *P. brassicae* benefits from BR and how it alters the synthesis in the plant should be a focus for future studies.

Lipids bodies were found in infected tissue. This shows that *P. brassicae* induce lipids in the plant roots. In non-infected plants, lipids are normally not found in root tissue. Since no fatty acid synthase is shown in the genome, *P. brassicae* might be dependent from the production and transport of host lipids (Schwelm et al. 2015). Siemens et al. (2006) found that *LTP1* was higher expressed during infection. We could not confirm this in clubroots, GUS expression of both *proLTP1a*- and *proLTP1b*-GUS reporter lines was not increased in clubroots. It might be possible that lipids are *de novo* synthesized in the clubs rather than transported. Further study is

needed to identify if genes involved in lipid biosynthesis are higher expressed in infected roots and clubroots. Until now, no GUS reporter strains are available for genes involved in lipid biosynthesis.

PR proteins are involved in the SA pathway that regulates the defense response against biotrophic pathogens. *P. brassicae* might suppress the defense response via a methyltransferase (PbBMST) (Ludwig-Müller et al. 2015). PbBMST is one of the highest expressed effector candidates in the host (Schwelm et al. 2015). Siemens et al. (2006) found that *PR1* was down-regulated during infection. We could not confirm this with altered GUS expression in the *proPR1*-GUS reporter line. We could not show an altered GUS expression in the *proPR2*-GUS genotype during *P. brassicae* infection either. Also for the JA related GUS-reporter lines, no markedly altered GUS expression was found during infection. JA regulates the defense response against necrotrophic pathogens. Agarwal et al. (2011) found up-regulation of *LOX4* during infection; we found only slightly higher GUS expression in *proLOX2*-GUS in infected roots. The biosynthesis of JA might be up-regulated. The JA and ethylene responsive *proPDF1.2*-GUS showed no differences in GUS activity in infected and non-infected roots, but GUS was highly expressed in the clubs indicating higher signaling of JA or ethylene in this tissue. JA induces glucosinolate activity which content is increased in the clubroots (Ludwig-Müller et al. 2009). Higher amount of JA may down-regulate the SA defense response. Both SA and JA defense pathways seems not to be induced during *P. brassicae* infection. *P. brassicae* might avoid plant defense responses.

5 Conclusion

This study showed that *A. thaliana* is a useful system to study the effect of *P. brassicae* during infection, but it is only possible to study this interaction using plants grown in soil. BR related genes showed higher GUS expression during infection. *P. brassicae* induces lipids in the roots. It is possible that lipids are *de novo* synthesized in the clubroots. Furthermore, SA and JA defense is not induced in infected tissue. How *P. brassicae* avoids the defense response and alters BR metabolism needs to be shown. Since the transcriptome of *P. brassicae* is known (Schwelm et al. 2015), it is possible to study the expression of *P. brassicae* effector candidates (PBRA genes) within the host by transforming *A. thaliana* plants. For BR, this has been initiated with transforming *A. thaliana* with a PBRA gene encoding for a protein with structural similarities to BRI1.

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