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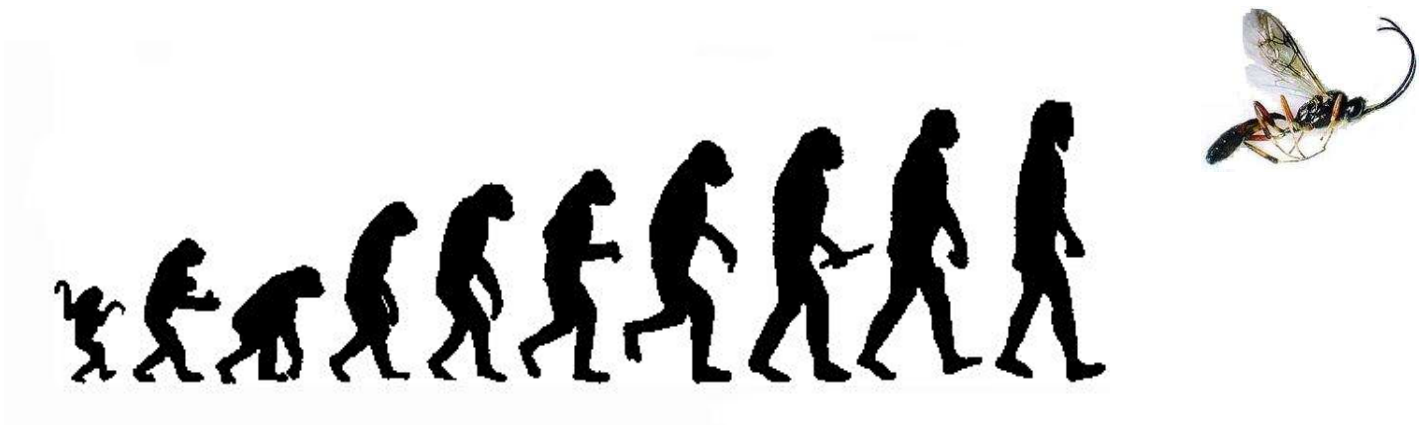
INDUCTION OF RESISTANCE BY NON-PATHOGENIC *PSEUDOMONAS FLUORESCENS* IN PLANTS (*BRASSICA OLERACEA* L.) UPON HERBIVORY OF INSECTS: DETERMINING DIRECT AND INDIRECT PLANT RESISTANCE IN A BEHAVIOURAL AND MOLECULAR APPROACH



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Abstract

Certain strains of plant growth-promoting rhizobacteria (PGPR), like *Pseudomonas fluorescens* are capable of suppressing diseases in plants by means of induced systemic resistance. Previous research concerned the suppression of diseases caused by pathogenic bacteria, fungi and nematodes in plants by these Pseudomonads. In this study, we focus on the tetratrophic interactions between PGPR, plants, herbivorous insects and a parasitoid.
Here, we demonstrate that *Pseudomonas fluorescens* strains CHA1144 and CHA0 are capable of reducing *P. rapae* larval weight after feeding on *B. oleracea*, which indicates that the plants direct defence is enhanced by the presence of specific non-pathogenic rhizobacteria in the soil. However, generalist *M. brassicae* was not effected by the presence of *Pf* CHA0 and the mutant strain CHA1144. In a Y-tube experimental set-up it was perceived that preference of parasitoid wasp *Diadegma semiclausum* for *P. fluorescens* CHA1144 infested plants was significantly higher compared to *P. fluorescens* CHA0 infested plants when exposed to *M. brassicae.* For *P.rapae* damaged plants *P. fluorescens* CHA1144 was preferred over control plants. The overall results showed a tendency towards the mutant strain for all comparison treatments. Gene expression study by quantitative real-time PCR showed no clear priming effect of both wild and mutant strains on plant induced defence in *B. oleracea* in current conditions.

Abbreviations*: ISR: Induced systemic resistance, SAR: Systemic Acquired Resistance, Pf: Pseudomonas fluorescens, PR: Pathogenesis-Related, ET: ethylene, SA: salicylic acid, JA: jasmonic acid.*

Contents

1.Introduction

Phytophagous insects are responsible for a major part of crop losses worldwide. Solutions to overcome this problem are found in applying pesticides and using biological control measures. One studied biological control method is the use of rhizobacteria from suppressive soils. Suppressive soils contain non-pathogenic rhizobacteria that are capable of suppressing pathogenic bacteria, fungi and nematodes (Siddiqui et al., 2005). Three different mechanisms contribute to this phenomenon: Aggressive root colonization of the PGPR, the production of antibiotic compounds that negatively effect pathogenic soil-borne organisms in the rhizosphere and the induction of systemic resistance in plants by means of ISR (Induced Systemic Resistance) (Haas et al., 2005; Keel et al., 2003; Knoester et al.,1999; Loon et al.,1998; Van Wees et al., 2000; Van Wees et al., 1999). Certain strains of plant growth-promoting rhizobacteria (PGPR), like *Pseudomonas fluorescens* are capable of suppressing diseases in plants. From the plant's point of view, non-pathogenic *Pf* bacteria cause plants to be in a unique physiological state: 'priming' (Conrath et al., 2006). Primed plants display either faster, stronger or both, activation of the various cellular defense responses that are induced by following attack of pathogens (Conrath et al., 2006, Pare et al., 2005). Although there is evidence on the induction of resistance in plants of certain *P.fluorescens* strains, leading towards a disease suppression of aboveground plant diseases caused by certain fungi and bacteria, the effect of PGPR on the suppression of insect herbivory is still unclear. Previous research concerning PGPR induced defence using *Arabidopsis thaliana* as a model plant exposed to *Pieris rapae* and *Spodoptera exigua* showed no effect in herbivore attractiveness of the parasitoid *Cotesia rubecula*. In order to verify the effect of ISR on *A. thaliana* direct defence mechanisms, bio-assay feeding experiments were accomplished and this revealed that the generalist *S. exigua* was physiologically impaired by the presence of PGPR (Van Oosten et al. 2007). In an RNA-blot analyses, defence-related genes *PDF1.2 and HEL* displayed an enhanced expression when challenged with *S. exigua* in the presence of *P. fluorescens* WCS417r (Van Oosten et al., 2007). In a novel approach to further extent this research, experiments with Brussels sprouts (*Brassicae oleracea L.*) were conducted in the presence of *P.fluorescens* strain WCS417r. *B.oleracea* root colonization by this strain resulted in a decrease of the fresh weight gain of generalist feeder *M.brassicae*, however no significant difference was observed in the weight gain of specialist *P.rapae* compared to the control. Experiments concerning indirect plant defences displayed a shift in parasitoid preference for both *P. rapae* and *M.brassicae* in wind tunnel and Y-tube behaviour bioassays. The effect of *P. fluorescens* WCS417r on expression of *BoLOX*, *BoMYC* and *BoPR1* after challenging cabbage plants with herbivores were confirmed by quantitative Real-time PCR (Zheng et al., unpublished results).

Plant defence against herbivorous insects

Herbivorous arthropods use different feeding modes, which can be piercing and sucking or tissue chewing. Tissue-chewing insects can instigate substantial leaf damage. Plants have developed various defence tactics to respond in an adequate and effective way upon insect attack, by defending themselves either by constitutive or induced defence. Constitutive defence can be structural or chemical, such as the presence of trichomes and toxic secondary metabolites. Constitutive investments in defence are costly, to overcome this problem plants utilize defence mechanisms induced upon insect attack (Van Poecke and Dicke, 2004).
Upon the release of regurgitant during insect herbivory, a signal processing is elicited in the plant followed by a cascade of gene expression and enzyme activity, resulting in for instance cell wall fortification and the production of plant signalling compounds. A limited number of phytohormones play a major part in this plant defence signalling, namely ethylene (ET), salicylic acid (SA) and jasmonic acid (JA) (Pieterse et al. 1998). Induced systemic resistance elicited by rhizobacteria involves JA and ET response in the signal-transduction pathway and thus non-pathogenic *Pf* strains are presumed to excite this particular signalling route. SA is produced in the systemic acquired resistance (SAR) signal transduction pathway elicited by biotrophic and hemitrophic pathogenic bacteria, while JA and ET are intermediates in resistance transduction in plants infested by necrotrophic pathogenic bacteria. Between plant defence pathways, an overlap in pathogenesis-related protein is detected in this web of complex responsive and produced defensive phytochemicals (Pieterse et al. 1998). Another overlap in plant resistance transduction pathways was shown for *Pf* strain CHA0, a PGPR, that coincides with *PR* gene accumulation during ISR. *PR* genes are markers in the SAR pathway. However, endogenous SA accumulation, a characteristic of the SAR pathway, was observed in CHA0 infested plants (Maurhofer et al. 1994). This *PR* expression was not detected by conventional Northern-blot when *A.thaliana* was challenged with *Pf* strain WCS417r (Pieterse et al., 1996). However, when *B. oleracea* was challenged with *Pf* strain WCS417r, an up-regulation in *PR1* expression by quantitative real-time PCR was observed compared to uninfested control plants (Zheng et al., unpublished results). Besides these major plant defence signal transduction pathways, several other pathways are employed by plants to cope with pathogen and insect attack.

Pathogenesis-related genes

In this study, five genes involved in different plant defence signalling pathways were examined to determine whether the presence of PGPR alters pathogenesis-related gene expression levels in *B. oleracea* upon *Pieris rapae* and *Mamestra brassicae* infestation. *B. oleracea* specific *PR1* is involved in the SAR pathway. The production of salicylic acid is a signal that elicits an up-regulation in *PR* expression levels and the production of pathogenesis-related proteins (PRs) (Pieterse et al., 1998). *PR1* accumulation in the presence of PGPR *Pf* wild type strain CHA0 and mutant strain CHA1144 might reveal that SAR and ISR pathways interconnect or depend on one another in *B. oleracea* defence. For *Pf* strain WCS417r it was recently observed that *BoPR1* expression was increased in primed cabbage leaves after herbivore damage (Zheng et al., unpublished results). Lipoxygenase encoded by *LOX* synthesizes jasmonic acid as part of the octadecanoid pathway and is up-regulated by leaf wounding (Moran et al., 2001). Similar to *LOX*, *B. oleracea* defensin gene *DEF*, is a part of the octadecanoid pathway. Insect herbivory leads to the production of linolenic acid in plants, which results in accumulation of JA and the activation of defence genes. A knock-out of *DEF* in plants results in the absence of JA and result in decreased insect resistance (Broekaert et al., 1995). *B. oleracea* MYC is a transcription factor, a protein that is homolog with *A. thaliana* MYC2. Knock out mutants *AtMYC* results in plants insensitive to JA (Boter et al., 2004). Similar to AtMYC, BoMYC functions as a JA-response transcription factor.

The plant defence-related enzyme myrosinase is encoded by *MYR*. Upon pest attack, cellular breakdown exposes metabolites called glucosinolates to degradative enzymes (myrosinases) (Bones and Rossiter, 1996). The myrosinase-glucosinolate system thereby produces degradation products that are involved in insect and pathogen resistance (Bones and Rossiter, 1996). Myrosinases, also called thioglucosidases catalyse the hydrolysis of glucosinolates. In that reaction glucose and sulphate are released and either nitrile, isothiocyanate, amine, epithionitrile, thiocyanate, oxazolidine-2-thione or other less prevalent products are formed (Rask et al., 2000; McGregor, 1988).

Plant growth promoting rhizobacteria (PGPR)

Two wild *P. fluorescens* strains and one derivate mutant strain were studied for their effect on plant defence against herbivorous insects. CHA0 (wild type), CHA1144 (ΔrsmXYZ; a transgenic mutant of CHA0) and WCS417r (wild type) were examined for their capability of suppressing larval growth of herbivorous insects and their effect on parasitoid behaviour.
Ample research has been conducted with CHA0 as a model stain for the biological control of fungal plant diseases (Siddiqui et al., 2005; Haas et al., 2002; Heeb et al., 2002; Keel et al., 2003). *Pf* CHA0 is able to suppress several plant pathogens such as the plant-pathogenic root-knot nematode *Meloidogyne incognita*, the fungus *Gaeumannomyces graminis* var. tritici, causal agent of take-all disease of wheat and *Thielaviopsis basicola*, a fungal pathogen causing black root rot of tobacco (Siddiqui et al., 2005; Haas and Keel., 2003; Laville et al., 1992). Experiments with multifarious CHA0 mutants showed that 2,4-diacetylphloroglucinol (2,4-DAPG), a secondary metabolite produced by *Pf* CHA0, is needed for the bacteria to induce ISR. It was observed that CHA631 (CHA0 [DAPG] deficient mutant) was the only mutation that led to a significant decrease in ISR and disease suppression when infected with oomycete *Peronospora parasitica* (Iavicoli et al., 2003). It was also observed that transgenic *A. thaliana* mutants insensitive to ethylene in the roots, insensitive to jasmonic acid and methyl jasmonic acid and mutants that were not expressing the NPR1 protein were unable to undergo ISR when infested with *Pf* CHA0 (Iavicoli et al., 2003).

CHA1144 is a CHA0 transgenic mutant lacking rsmXYZ. RsmX, RsmY and RsmZ are RNA genes that are positively controlled by a GacS/GacA (acronym for Global Activator of Antibiotic and Cyanide synthesis) (Haas and Keel, 2003) control system, leading to transcription of these genes into small regulatory RNAs (Kay et al., 2005).
Subsequently, these genes bind RsmA and RsmE in *P. fluorescens*, responsible for repression of translational processes of secondary metabolites. ΔCHA1144 does not synthesize these small RNA genes and is therefore not capable of binding translation repressors RsmA and RsmE, resulting in repression of translational and posttranslational biocontrol processes. Various secondary metabolites, like phenazine-1-carboxylate, DAPG, pyrrolnitrin, phenazine-1-carboxamide, pyocyanine, hydrogen cyanide and viscosinamide are not produced in the transgenic mutant strain, of which DAPG is a crucial instigator of ISR. *Pf* CHA1144 is impaired in certain properties like swarming motility and the synthesis of quorum-sensing signals when tested for suppression of the fungal pathogen *Phytium ultimum* in cucumber (Kay et al., 2005; Reimmann et al., 2005; Haas and Keel, 2003).

The role of *Pf* WCS417r as an inducer of ISR is well studied as an effective biocontrol agent against the bacterial leaf pathogen *P. syringae* pv tomato, the fungal root pathogen *Fusarium oxysporum sp raphani* and the fungus *Peronospora parasitica* (Leon-Kloosterziel et al., 2005; Pieterse et al.,1997; Pieterse et al., 1996; van Wees et al., 1997). Molecular analysis showed that *Pf* WCS417r causes a considerable modification in the expression of almost 100 genes (Leon-Kloosterziel et al., 2005; Verhagen et al., 2004). Mutant analysis displayed that WCS417r-mediated ISR requires an intact response to JA and ET (Pieterse et al., 1998). Experiments on the effect of *Pf* WCS417r on SAR previously showed that the strain does not induce SAR in plants (Verhagen et al., 2004; Van Wees et al., 1999; Ton et al., 2002; Ton et al., 2001).

In this study, I perform experiments on tetratrophic interactions between belowground *Pf* stains, the aboveground host plant, herbivorous insects and a parasitoid wasp. Both an ecological approach, larval weight gain and Y-tube olfactometer experiments, and a molecular approach, quantitative Real-Time PCR, are combined to obtain in depth information on these interactions.

Research questions

Research questions are raised in this project indicated as below.

Is there an effect of priming for gene expressions due to wild type strain *Pf* CHA0 and the derivate mutant *Pf* CHA1144? *Quantification of gene expression by Quantitative Real-Time PCR.*

Which pathways are involved in PGPR infested *B. oleracea* L. defence against herbivore predation*?* The complexity of the plant defence mechanism studied with the use of molecular tools. *Quantifying the expression levels of several genes involved in various plant defense signaling pathways.*

Does priming influence the direct plant defense and plant damage? *Measuring relative larval body weight gain of specialist feeder P. rapae and generalist feeder M. brassicae.*

Does priming influence the indirect plant defense against herbivores? *Conducting Y-tube experiments to examine altered parasitoid behavior of D. semiclausum. Quantifying genes involved in indirect defence by Quantitative Real-Time PCR.*

2.Materials and Methods

2.1 Body weight experimental set-up

In the first part of the project, the fitness of the specialist small cabbage white butterfly (*Pieris rapae* L.) and the generalist cabbage moth (*Mamestra brassicae* L.) was tested. Both herbivorous insects were tested on *Brassica oleracea* L. in a bio-assay experiment for relative weight gain on *P. fluorescens* infested and uninfested plants.

2.1.1 Growing bacterial strains

P. fluorescens strains CHA0, WCS417r and CHA1144 were plated on solid King's B medium (King 1954) (**picture 2.1b**; appendix I) and grown at 28°C for 2-4 days. Additionally, *P. fluorescens* WCS417r was grown in the presence of the antibiotic rifampicin (25 µg/ml). For *P. fluorescens* the strains CHA0, CHA1144 (provided by Prof. Dieter Haas, University of Lausanne, Switzerland) and WCS417r (provided by Prof. Corné Pieterse, Utrecht University, The Netherlands) were adjusted to a concentration of OD660=1.0 at a density of 10^9 cfu/ml in a 10mM $MgSO_4$ solution.

2.1.2 Soil preparation

Potting soil and regular yellow sand were mixed in a 4:1 proportion, respectively. The mixed soil was autoclaved for two times 15 minutes at 120°C. Per kg of soil, 50ml of the bacterial suspension was added and mixed. For a control, 50ml of MgSO4 suspension per kg of soil was added and mixed.

2.1.3 Preparing Brassica oleracea L.

Four-week old Brussels sprouts plants with two true leaves (*Brassica oleracea* var. gemmifera cv. Cyrus) seedlings were provided by Unifarm (**picture 2.1a**; appendix I). The seedlings were transferred to the prepared soil and grown for another two weeks (**picture 2.1c**; appendix I). Plants were watered on regular basis and enriched with 1 ml fertilizer per plant once a week (growing conditions 20-30 ºC, RH 50-70%, 16L: 8D).

2.1.4 Preparing Pieris rapae and Mamestra brassicae

Both *P. rapae* and *M. brassicae* were reared at room temperature (20-30 ºC, RH 50-70%, 16L: 8D). Rearing of *P. rapae* eggs and neonate larvae was done on *B. oleracea* plants. Rearing of eggs and neonate larvae of *M. brassicae* was done on filter paper. Per plant, five freshly emerged 1st instar larvae were transferred to the leaves of *B.oleracea* with a moist brush. After transfer of the larvae, body weight was measured from the onset of feeding until eight days post relocation. Final weight represented the total weight gain since the starting weight of the larvae was presumed to be insignificant. Larvae were weighted on a microbalance.

2.1.5 Statistical data analysis

Data was analysed with the GenStat 8.11 statistical program, using a generalized linear model. T-probabilities of pairwise differences were used to compare weight gains among treatments.

2.2 Behavioural essay

The Y-tube olfactometer experiment was contrived to test preference of the parasitoid *D. semiclausum* for different *Pf* treatments. The hypothesis comprehends that increased oviposition behaviour of female parasitoids occurs when the plant 'cries for help' by means of excreting defence volatiles. It was expected that *P. fluorescens* CHA0 and WCS417r infested soil led to an increased production of defense volatiles, by means of ISR induction, resulting in increased oviposition behaviour of *D. semiclausum.*

2.2.1 Principal of Y-Tube method

Picture 2.2 (Appendix I) outlines the Y-tube setting. As an air flow (four bars per pot) was generated through the tubes towards *D. semiclausum*, the parasitoid was attracted by emitted plant defence volatiles.

A choice occurred when individual *D. semiclausum* wasps crossed a line within ten minutes and did not return to the junction for at least fifteen seconds. If the junction was not reached within five minutes, the parasitoids were considered as non-responding individuals. For the experimental set-up, binary tests were conducted as indicated in **table 2.1**.

2.2.2 Rearing of Diadegma semiclausum
D. semiclausum cocoons were laid on cardboard paper. Newly emerged wasps were collected and transferred to a grow chamber (23°C; 16L:8D). As a food and liquid source, honey and moist filter paper were provided. Five to ten days old *D. semiclausum* were sexed and females were selected.

2.2.3 Preparing Brassica oleracea L.
Four-week old *B. oleracea* var. gemmifera cv. Cyrus seedlings were provided by Unifarm as described as before. The seedlings were transferred to the prepared soil and grown for another two weeks. Plants were watered on regular basis and enriched with fertilizer once a week.

2.2.4 Preparing P. rapae and M. brassicae
Both *P. rapae* and *M. brassicae* were reared as described as before. Per plant, five 1st instar larvae were transferred to the leaves with a moist brush. Plants were used in the Y-tube experiments 24h after the larvae were transferred to the plant.

2.2.5 Statistical data analysis
Data was analysed in Microsoft Excel with a binominal distribution test. The expected distribution was 0.5, assuming a normal distribution.

Table 2.1 Different treatments for the Y-tube olfactometer experiment with left treatment vs. right treatment in a binary equation.

Left treatment	versus	Right treatment
P. fluoresens CHA0 treated plant infested by *P. rapae*		Untreated plant infested by *P.rapae*
P. fluoresens CHA0 treated plant infested by *M. brassicae*		Untreated plant infested by *M.brassicae*
Untreated plant infested by *M. brassicae*		Untreated plant infested by *P. rapae*
P. fluoresens CHA0 treated plant infested by *M. brassicae*		*P. fluoresens* CHA0 treated plant infested by *P. rapae*
P. fluoresens CHA1144 treated plant infested by *P. rapae*		Untreated plant infested by *P. rapae*
P. fluoresens CHA1144 treated plant infested by *M. brassicae*		Untreated plant infested by *M. brassicae*
P. fluoresens CHA1144 treated plant infested by *M. brassicae*		*P. fluoresens* CHA1144 treated plant infested by *P.rapae*
P. fluoresens CHA0 treated plant infested by *P. rapae*		*P. fluoresens* CHA1144 treated plant infested by *P. rapae*
P. fluoresens CHA0 treated plant infested by *M. brassicae*		*P. fluoresens* CHA1144 treated plant infested by *M. brassicae*

2.3 Molecular analyses by Real-Time PCR

2.3.1 Principle of Real time PCR method

To determine expression levels of specific *B. oleracea* defence-related genes when exposed to different treatments (**table 2.2**), a quantitative Real-Time PCR was conducted. These experiments were performed at Plant Research International (PRI).

Figure 2.1 explains the main principle of the Real-Time PCR. As stated in **table 2.3.**, five genes involved in plant defense and a reference gene were tested. Upon expression of genes mRNA can be isolated and transcribed into cDNA by reverse transcription. When using gene specific primers during amplification, the fluorogenic dye SYBR green binds to double stranded DNA and becomes fluorescent upon binding. Fluorescence is detected by the Real-Time PCR Detection System and expression levels can be quantified.

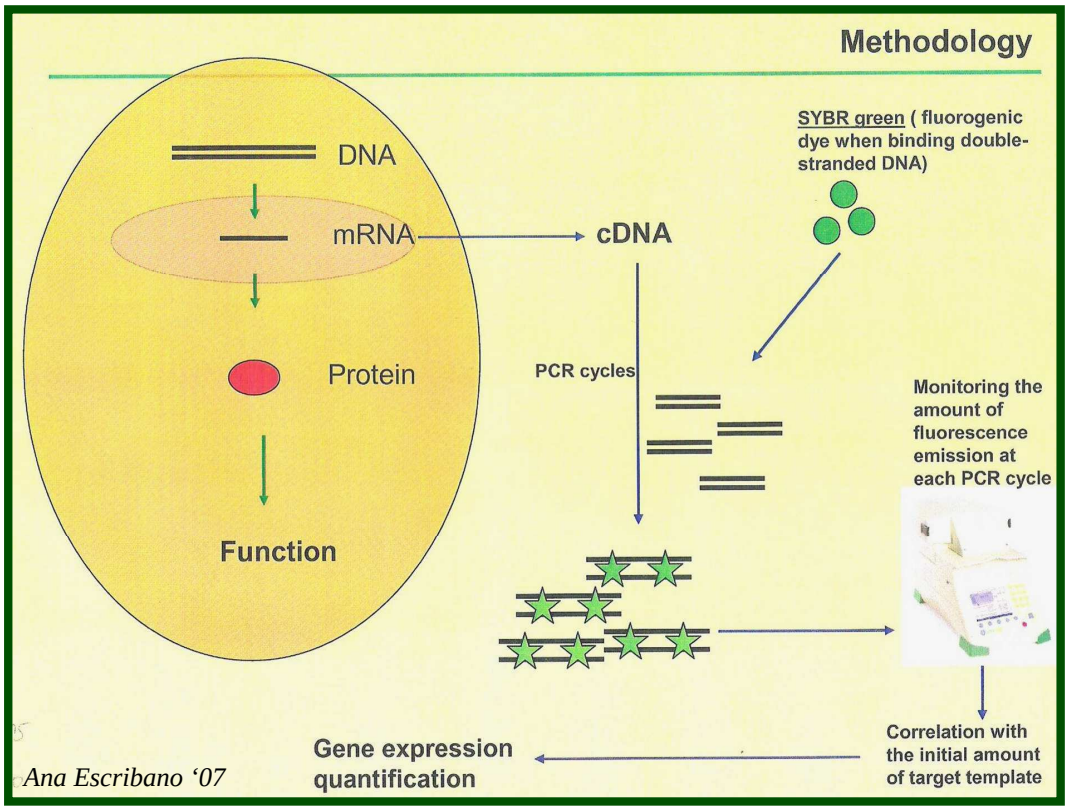

Figure.2.1. Real-Time PCR principle: mRNA levels in the cell increase when a gene is induced. mRNA will be converted into cDNA by RT-PCR. The fluorescent dye SYBR green binds to dsDNA and fluorescence is measured at each PCR cycle. Gene expression can be quantified by calculating the normalized expression.

2.3.2 Plant material collection

Whole leaves were collected from *B. oleracea* for the different treatments after zero and six hours of *P. rapae* and *M. brassicae* infestation (**table 2.2**). Clip cages were used to avoid migration of the caterpillar to systemic leaves (**picture 2.3;** appendix I**)**. Both local and systemic leaves were included (**table 2.2**). The leaf situated above the local leaf was denoted as the systemic leaf. Per sample a whole leaf was wrapped in aluminum foil and transferred to liquid nitrogen. Leaves were stored at minus 80°C.

2.3.3 RNA isolation

Total RNA isolation was done at PRI by using chemical fluid TRIzol (Invitrogen). The plant tissue was grinded in liquid nitrogen until fine powder remained. 3ml TRIzol was added to a blue cap tube and mixed with the plant material by vortexing. Chloroform (0.2 ml chloroform:1ml TRIzol) was added and mixed by vortexing. The suspension was transferred to centrifuge tubes and centrifuged (20 min; 4°C; 4600rpm). The aquarious phase was transferred to a new centrifuge tube and an equal volume of isopropanol was added. The material was centrifuged (15 min; 4°C; 4600rpm). The pellet was kept and cleaned with 75% ethanol and centrifuged (7 min; 4°C; 4600rpm). Pellet was dried and solved in 200µl of RNase free water.

2.3.4 Determining RNA purity

Total RNA concentration and purity was measured with a NanoDrop_ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA). The RNA solution was stored at minus 80°C. The quality of the RNA samples were assessed using 1.0% agarose gel electrophoresis (1,5h 50V) and ethidium-bromide (0.1µl per ml TBE buffer) visualization. RNA was visualized under a uv-radiation light.

2.3.5 RNA purification and DNase treatment

RNA purification was done using an RNeasy Mini-elute kit from Qiagen according to the manufacturer's protocol. After the purification steps, the RNA concentration and purity was measured with a NanoDrop_ ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA). The purified RNA was treated with 10x Dnase1 (Invitrogen) in a reaction buffer (RT; 15 min.). After adding 1µl of EDTA (25mM), the mix was incubated in a thermo-cycler at 65°C for 10 min.

2.3.6 Quantitative Real-Time PCR

A mix of Oligo (dT) and hexamer primers (Biorad) was used for the first-strand cDNA synthesis implementing the reverse transcription protocol and an iScript cDNA synthese kit (Biorad; according to the manufacturer's instructions). A master mix consisting of 22.5µl iQ SYBR green supermix (Biorad), 4.5µl forward and reverse primers (3µM) and 4.5µl MQ water per duplicate was prepared. RT-PCR reactions were performed in optical 96-well plates with MyiQ™ Single Color Real-Time PCR Detection System (Biorad). Primer sets were used to detect the relative expression of several defence genes (**table 2.3**). As a reference gene the constitutive expressed gene glyceraldehyde-3-phosphate-dehydrogenase (*GADPH*) was included (*GADPH* forward primer: 5'-AGA GCC GCT TCC AAC ATC ATT-3'; *GADPH* reverse primer: 5'-TGG GCA CAC GGA AGG ACA TAC-3'). DEPC water was included as a negative control. Five *B. oleracea* specific genes were used (*BoDEF* forward primer: 5'-GTT TGC TTC CAT CAT CAC CCT TCTC-3'; *BoDEF* reverse primer: 5'-CAC TTG ACC TCT CGC ACA ACT TAG-3')(*BoPR1* forward primer: 5'-GTC AAC GAG AAG GCT AAC TAT AAC TACG-3'; *BoPR1* reverse primer: 5'-TTA CAC CTT GCT TTG CCA CAT CC-3')(*BoLOX* forward primer: 5'-ACT TTC CCG TCC CGT TCT TGG-3'; *BoLOX* reverse primer: 5'-GAT TGT CGT GCC CGT GAA TGC-3')(*BoMYR* forward primer: 5'-GTT TCT TAG ACC GCC AGA TCA TAC AAG-3'; *BoMYR* reverse primer: 5'-CAG TGC TTT ACC TTT CCA CCA AAT TC-3')(*BoMYC* forward primer: 5'-GGC TGG ACC TAC GCT ATA TTC TGG-3'; *BoMYC* reverse primer: 5'-GCT CAC GCA ACA CCT TCT TAGC-3'). The following PCR program was used for all PCR reactions: 95°C for 3 min; 40 cycles of 95°C for 30 sec and 60°C for 45 sec. C_T (threshold cycle) values were calculated using Optical System Software, version 2.0 for MyIQ (Biorad) (Broekgaarden et al., 2007).

2.3.7 Statistical data analysis

Data analysis was performed with expression tables (**figure 2.4;** appendix I), melt curve peak charts (**figure 2.3;** appendix I) and amplification charts (**figure 2.2;** appendix I) with MyiQ analyses software (Biorad). Data was normalized with the reference gene (2^-(delta Ct(Ctgene-Ctref)) and was analysed with the GenStat 8.11 statistical program, using a generalized linear model for normal distribution. Normalized values were converted to log e values. T-probabilities of pairwise differences were used to detect significant differences between treatments.

Table 2.2. Treatments used in Quantitative Real-Time PCR

Sample No.	Treatment	Period of larval exposure
1	*P. fluoresens* CHA0 treated plant infested by *P. rapae* – local	6h
2	*P. fluoresens* CHA0 treated plant infested by *P. rapae* -- systemic	6h
3	*P. fluoresens* CHA0 treated plant infested by *M. brassicae* -- local	6h
4	*P. fluoresens* CHA0 treated plant infested by *M. brassicae* -- systemic	6h
5	*P. fluoresens* CHA0 treated plant uninfested	0h
6	*P. fluoresens* CHA1144 treated plant infested by *P. rapae* -- local	6h
7	*P. fluoresens* CHA1144 treated plant infested by *P. rapae* -- systemic	6h
8	*P. fluoresens* CHA1144 treated plant infested by *M. brassicae* -- local	6h
9	*P. fluoresens* CHA1144 treated plant infested by *M. brassicae* -- systemic	6h
10	*P. fluoresens* CHA1144 treated plant uninfested	0h
11	Untreated plant infested by *M. brassicae* -- local	6h
12	Untreated plant infested by *M. brassicae* -- systemic	6h
13	Untreated plant infested by *P. rapae* -- local	6h
14	Untreated plant infested by *P. rapae* -- systemic	6h
15	*P. fluoresens* untreated and uninfested plant	0h

Table 2.3. Reference and plant defence-related genes used for quantitative Real-Time PCR.

Gene	Protein	Function
BoLOX	*Brassica oleracea* lipoxygenase	Induced defense and part of JA pathway (Siedow, 1991; Zheng et al., 2007)
BoMYC	*Brassica oleracea* transcription factor	JA transcription factor (Boter et al. 2004)
BoPR1	*Brassica oleracea* pathogenesis-related protein1 (PR1)	Marker gene of SAR (Van Wees et al. 2000)
BoDEF	*Brassica oleracea* defensin protein	Part of JA pathway (Siedow, 1991)
BoMYR	*Brassica oleracea* myrosinase	Catalyses glucosinolates. Products are involved in insect resistance (Rask et al., 2000; McGregor. 1988)
GADPH	Glyceraldehyde-3-phosphate-dehydrogenase	Housekeeping gene; constitutive expressed gene.

3.Results

3.1 Direct defence induced by P. fluorescens

To determine whether priming influences direct defence mechanisms in *B. oleracea* upon caterpillar feeding, either by up-regulating or down-regulating defence-related plant genes, a larval body weight experiment was designed. Both specialist feeder *P. rapae* and generalist feeder *M.brassicae* were examined for their body weight gain when consuming plants exposed to three different *Pf* strains: CHA0, CHA1144 and WCS417r. Per *Pf* treatment eight *B. oleracea* replicas were used with on each plant five neonate larvae. Larvae were weighted and T probabilities of pairwise differences (GenStat 8.11) were used to determine whether there is a significant difference of larval weight when feeding on control and PGPR primed plants.

Results were shown for weight gain of the generalist *M. brassicae* when feeding on *B.oleracea* plants for eight days in **table 3.1.** All strains tested did not seem to influence the feeding behavior of *M. brassicae* resulting in altered larval body weight (P>0.05). It indicates that larval weight gain is unaffected by the presence of PGPR (**figure 3.1**).

The transfer of *M. brassicae* neonate larvae to plants is difficult, because larvae do not stick to the leaves and easily fall of. Despite this almost all larvae were found on the plants after eight days of feeding. This might indicate that there was little roaming around of the larvae and also that larvae did not move in between plants.

Table 3.1. Differences in weight gain of generalist *M.brassicae* between control plants treated with MgSO4 or plants treated *Pf* strains CHA1144, CHA0 and WCS417r (T probabilities of pairwise differences).

Treatments	Control + *M. brassicae*	*Pf* CHA0 + *M.brassicae*	*Pf* CHA1144 + *M.brassicae*	*Pf* WS417r + *M.brassicae*
Control + *M.brassicae*	-	0.894	0.757	0.904
Pf CHA0 + *M.brassicae*	0.894	-	0.857	0.987
Pf CHA1144 + *M.brassicae*	0.757	0.857	-	0.847
Pf WS417r + *M.brassicae*	0.904	0.987	0.847	-

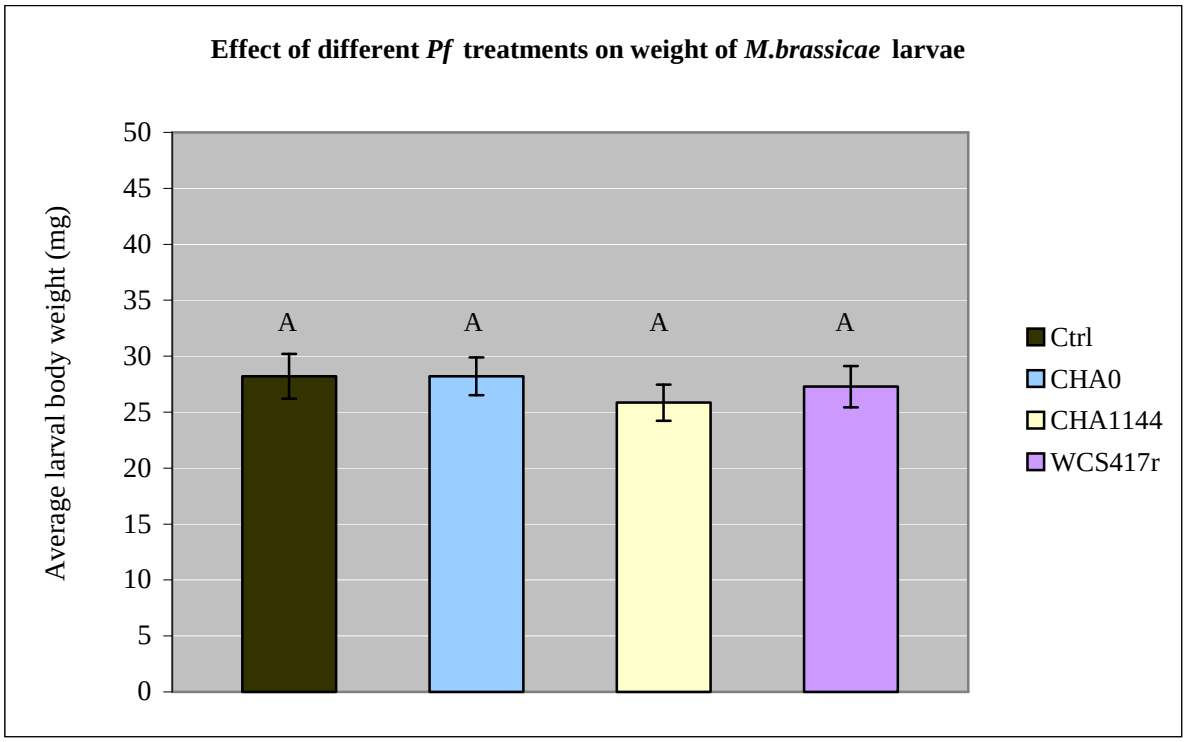

Figure 3.1. *Pf* strains CHA0 (light blue), WCS417r (purple) and CHA1144 (bright yellow) were tested for their effect on direct plant defence against the larvae of *M.brassicae*. As a control (dark green) MgSO4 was used. Indicated is the average larval body weight after eight days of feeding on *B.oleracea*. Error bars indicate SE of mean.

The results for *P.rapae* body weight gain after eight days of feeding on control and primed *B. oleracea* plants are presented in **figure 3.2**. *P. rapae* larvae that fed on plants infested with wild type strain WCS417r displayed no significant different body weight when compared to the control treatment (P>0.05). The *Pf* CHA0 treatment resulted in a highly significant decline in larval body weight compared to the control treatment (P<0.05) (**table 3.2**), no significant difference was found when *Pf* strains CHA0 and WCS417r are compared (P>0.05). Larvae that had been eating from plants treated with transgenic mutant *Pf* strain CHA1144 showed a highly significant (P<0.05) decline in average body weight when equated to both the control and the *Pf* WCS417r treatment.

The transfer of *P. rapae* neonate larvae to *B. oleracea* plants is efficient, because larvae stick to the plants and do not fall of. Despite this knowledge, not all larvae were found back eight days after the onset of the experiment. Also it was observed that larvae of the specialist *P. rapae* have the tendency to move in between plants. When collecting the larvae, more larvae than the initial amount of larvae were present on some plants while fewer larvae were found on other plants.

Table 3.2. Differences in weight gain of specialist *P. rapae* between control plants treated with MgSO4 or plants treated *Pf* strains CHA1144, CHA0 and WCS417r (T probabilities of pairwise differences)

Treatment	Control + *P. rapae*	*Pf* CHA0 + *P. rapae*	*Pf* CHA1144 + *P. rapae*	*Pf* WCS417r + *P. rapae*
Control + *P. rapae*	-	0.002 ***	0.000 ***	0.214
Pf CHA0 + *P. rapae*	0.002 ***	-	0.338	0.057
Pf CHA1144 + *P. rapae*	0.000 ***	0.338	-	0.004***
Pf WCS417r + *P. rapae*	0.214	0.057	0.004***	-

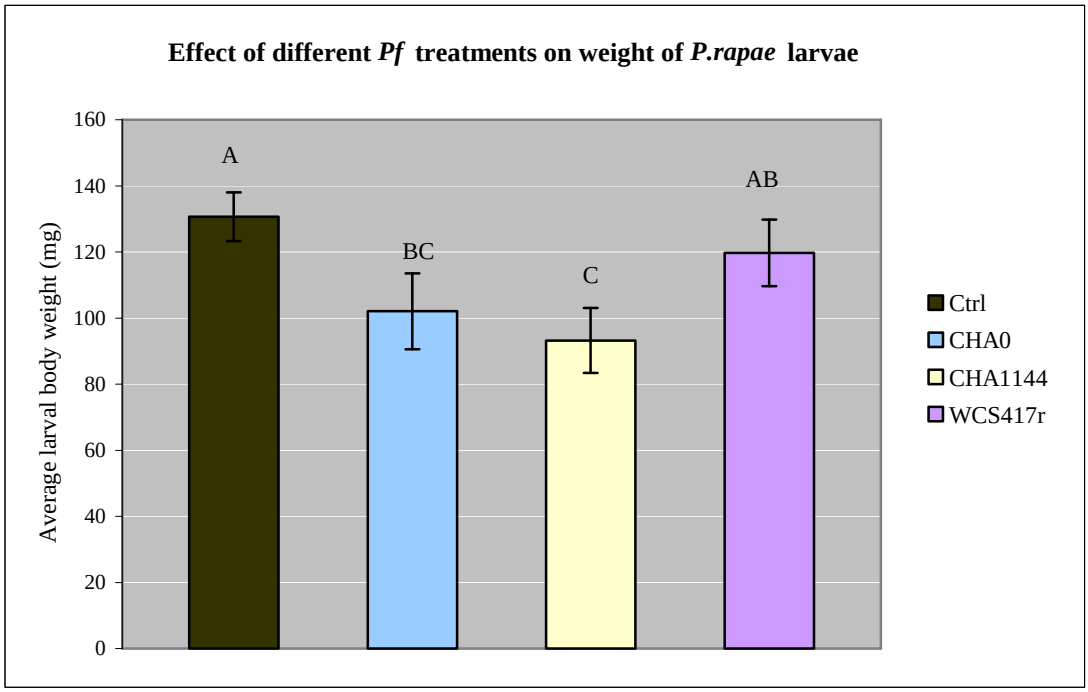

Figure 3.2. *Pf* strains CHA0 (light blue), WCS417r (purple) and CHA1144 (bright yellow) were tested for their effect on direct plant defence against the larvae of *P.rapae*. As a control (dark green) MgSO4 was used. Indicated is the average larval body weight after eight days of feeding on *B.oleracea*. Error bars indicate SE of mean.

3.2 Indirect defense induced by P. fluorescens

To study whether the presence of *Pf* in the soil results in an altered indirect defense mechanism of plants, a Y-tube experiment was designed. In this experiment the parasitoid *D. semiclausum* was offered the choice between plants treated with either *Pf* CHA0, *Pf* transgenic triple mutant CHA1144 or control MgSO4 treated. Five larvae of *P. rapae* or *M. brassicae* were transferred to the plants 24 h prior to the test (**table 2.1.**). Between sixty and eighty *D. semiclausum* wasps were tested for their plant preference per treatment in a binary comparison. In order to provide substantial results from a biological point of view the tests were dispersed over a considerable period of three months.

Results for preference of *D. semiclausum* for the different treated plants exposed to *P. rapae* were shown in **table 3.3** and **figure 3.3**. In the two choice experiment between plants grown in the presence of CHA0 and control, no significant difference was found (P>0.05). In total 31.67% of the wasps failed to make a choice within ten minutes after release. There was no tendency observed between the different test days towards a specific treatment. A highly significant difference (P<0.05) was seen for plants grown in the presence of CHA1144 and control plants. *D. semiclausum* strongly prefers plants that were infested with the transgenic mutant strain. A strong preference for *Pf* CHA1144 was seen for every individual test day.

No significant difference was observed for the *Pf* CHA0 and CHA1144 treatments (P>0.05). The overall preference of *D. semiclausum* tends to be towards CHA1144. This is not validated when various daily test results are considered. The percentage of wasps that failed to make a choice is significantly (P=0.022; not shown) smaller for the CHA0 and CHA1144 treatment compared to the CHA0 and control treatment.

Table 3.3. *D.semiclausum* preference for plants treated with CHAO, CHA1144 and MgSO4 exposed to *P. rapae*. The second and the third columns show the two treatments in the binary comparison. The fourth column shows the number of wasps that were not included as no choice was made within ten minutes. The last column shows the results of the statistical analyses done with a MS Excel binominal distribution test.

Exp.	Treatment 1	Treatment 2	# No choice	Total # tested wasps	% No choice	Binominal distribution
Date	**CHA0 *P.rapae***	**Ctrl *P. rapae***				
17-4-2007	8	9	3	20	15	0.5000
21-3-2007	6	4	10	20	50	0.8281
20-4-2007	6	8	6	20	30	0.3953
Total	20	21	19	60	31.67	P= 0.50000
Date	**Ctrl *P.rapae***	**CHA1144 *P.rapae***				
17-4-2007	7	14	0	21	0	0.0946
22-5-2007	1	14	5	20	25	0.0005
23-5-2007	2	14	4	20	20	0.0021
Total	10	42	9	61	14.75	P= 4.53164E-6 ***
Date	**CHA0 *P.rapae***	**CHA1144 *P.rapae***				
29-3-2007	10	8	2	20	10	0.7597
22-5-2007	7	7	6	20	30	0.6047
26-6-2007	8	18	4	30	13.33	0.0021
Total	25	33	12	70	17.42	P= 0.17907

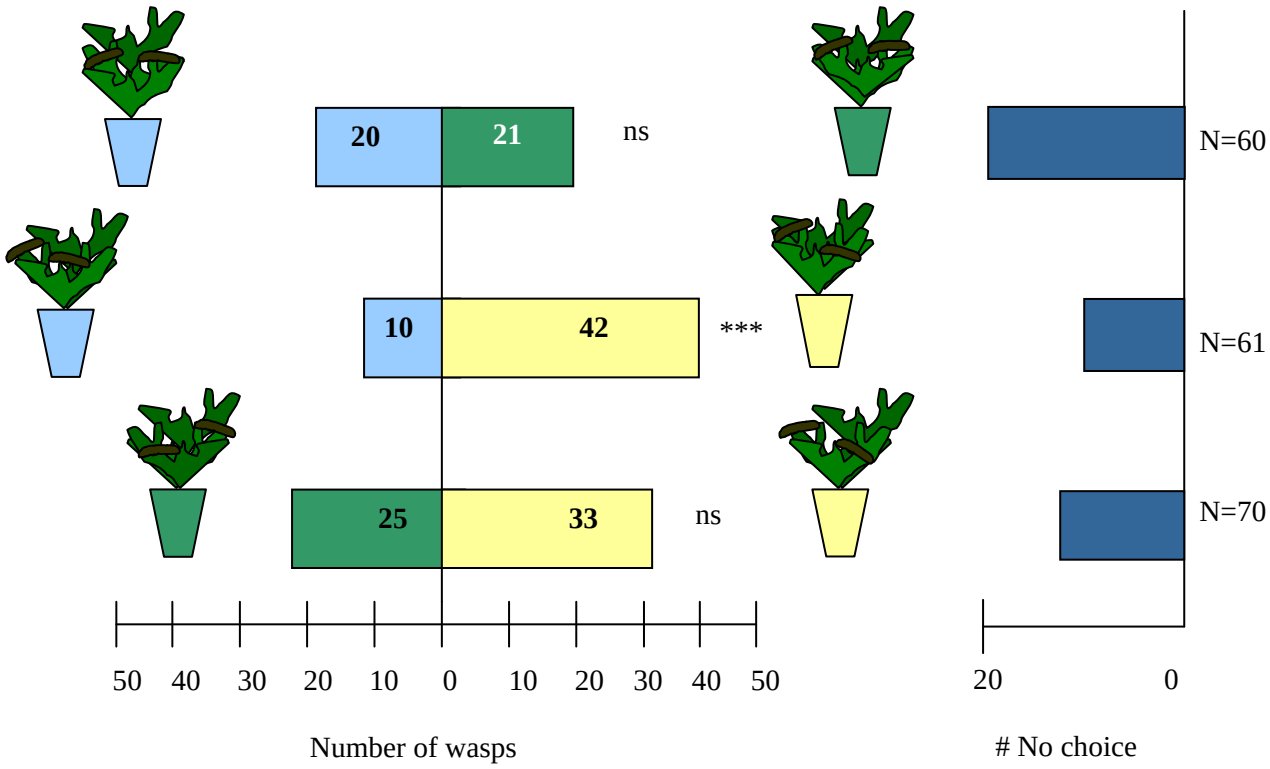

Figure 3.3. *D.semiclausum* response for different *Pf* treatments after 24h exposure of *P.rapae* on *B.oleracea.* Control treatments and *Pf* strains CHA0 and CHA1144 treatments are indicated with light green, blue and yellow respectively. Figures displayed in the columns define the total number of wasps that chose a specific treatment.

The results for the preference of *D.semiclausum* for plants exposed to generalist feeder *M.brassicae* were shown in **table 3.4** and **figure 3.4**. There is no significant difference (P>0.05) when comparing *Pf* strain CHA1144 to the control. There is a tendency of *D.semiclausum* preference towards CHA1144 treated plants in the overall results from The 2nd and 3rd column of **table 3.4**. Two out of three daily test results indicate that the parasitoid has a strong preference for *Pf* CHA1144. In the two choice experiment between plants grown in the presence of CHA0 and control, no significant difference was found (P>0.05). In total 21.67% of the wasps failed to make a choice within ten minutes after release. There was no tendency seen between the different test days towards either CHA0 or control treated plants. A highly significant difference (P=0.004) was observed for the *Pf* CHA0 and CHA1144 treatments. The 2nd and 3rd column define that on each individual test day, *D. semiclausum* prefers CHA1144 infested plants. The overall picture shows that the trend is towards plants infested with *Pf* CHA1144.

Table 3.4. *D.semiclausum* preference for plants treated with CHA0, CHA1144 and MgSO4 exposed to *M.brassicae*. The second and the third columns show the two treatments in the binary comparison. The fourth column shows the number of wasps that were not included as no choice was made within ten minutes. The last column shows the results of the statistical analyses done with a MS Excel binominal distribution test.

Exp.	Treatment 1	Treatment 2	# No choice	Total # tested wasps	% No choice	Binominal distribution
Date	**CHA1144 *M. brassicae***	**Ctrl *M.brassicae***				
24-4-2007	13	6	1	20	5	0.9682
19-6-2007	11	6	3	20	15	0.9283
26-6-2007	8	11	1	20	5	0.3238
Total	32	23	5	60	8.33	P= 0.14030
Date	**CHA0 *M.brassicae***	**CHA1144 *M.brassicae***				
30-3-2007	2	13	5	20	25	0.0037
18-4-2007	7	9	4	20	20	0.4018
19-6-2007	6	12	2	20	10	0.1189
Total	15	34	11	60	18.33	P= 0.00470 **
Date	**CHA0 *M.brassicae***	**Ctrl *M.brassicae***				
23-3-2007	8	8	4	20	20	0.5981
5-6-2007	12	5	3	20	15	0.9755
6-6-2007	7	7	6	20	30	0.6047
Total	27	20	13	60	21.67	P= 0.19085

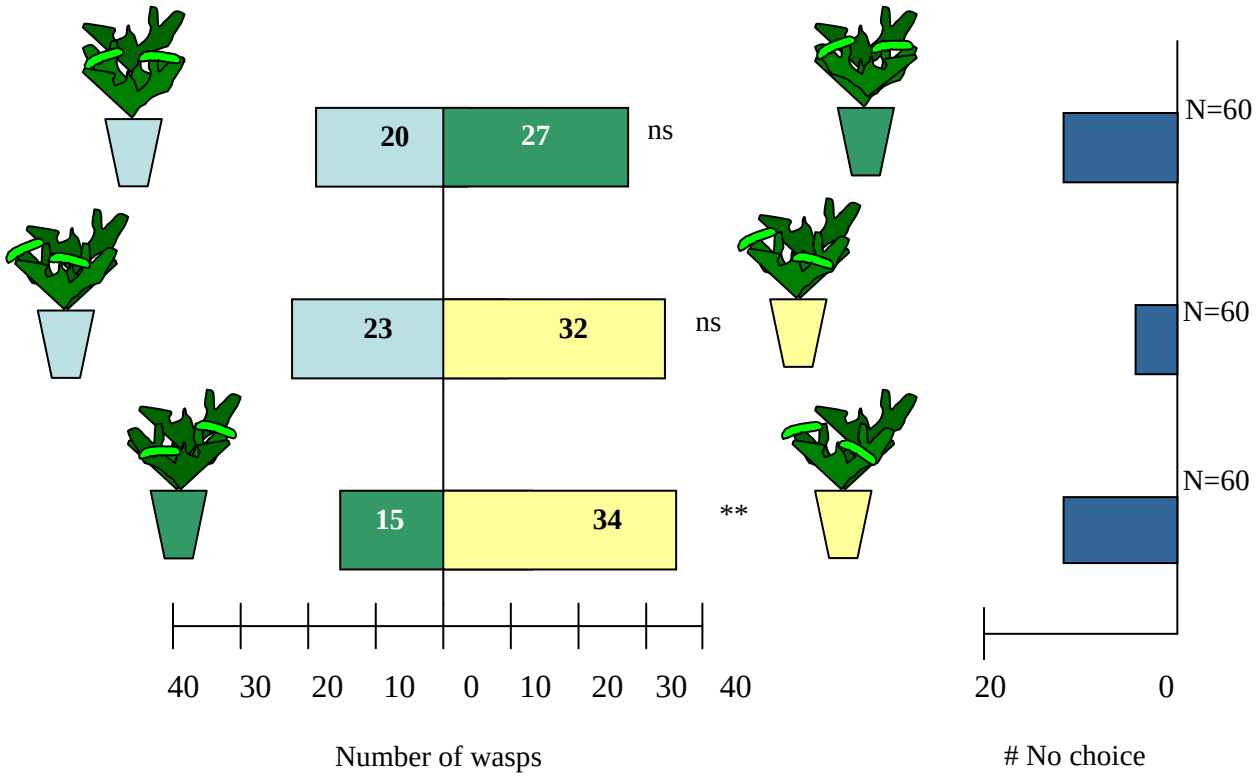

Figure 3.4. *D.semiclausum* response for different *Pf* treatments after 24h exposure of *M.brassicae* on *B.oleracea.* Control treatments and *Pf* strains CHA0 and CHA1144 treatments are indicated with light green, blue and yellow respectively. Figures displayed in the columns define the total number of wasps that chose a specific treatment.

Wasp *D.semiclausum* was also offered the choice between plants treated with a similar PGPR or control soil treatment (CHA0, CHA1144 or control), exposed to both larvae species *P.rapae* and *M.brassicae* in **table 3.5** and **figure 3.5**. There is a preference of the parasitoid *D.semiclausum* for plants exposed to *P.rapae* for both control and CHA1144 infested plants ($P<0.05$) (**table 3.5)**. The preference for *P.rapae* on CHA1144 infested plants is observed for every individual testing day. For the control treated plant it is observed that leaf damage elicited by *P.rapae* is preferred by the wasp during three out of four individual tests and tested significant in the integral results. No significant choice was made by the parasitoid wasp for CHA0 infested plants exposed to both *P.rapae* and *M.brassicae* ($P>0.05$). However, the tendency of parasitoid *D.semiclausum* is towards *P.rapae* damaged plants in the overall results and in the results of two out of three individual test days.

Table 3.5. *D.semiclausum* preference for plants treated with CHAO, CHA1144 and MgSO4 exposed to *P.rapae* and *M.brassicae*. The second and the third columns show the two treatments in the binary comparison. The fourth column shows the number of wasps that were not included as no choice was made within ten minutes. The last column gives the results of the statistical analyses done with a MS Excel binominal distribution test.

	Treatment 1	Treatment 2	# No choice	Total # tested wasps	% No choice	Binominal distribution
Date	**CHA0 *P.rapae***	**CHA0 *M.brassicae***				
23-3-2007	11	7	2	20	10	0.8811
25-4-2007	6	9	5	20	25	0.3036
5-6-2007	13	4	3	20	15	0.9936
Total	30	20	10	60	16.67	P= 0.10132
Date	**Ctrl *M.brassicae***	**Ctrl *P.rapae***				
5-6-2007	8	9	3	20	15	0.5
6-6-2007	8	6	6	20	30	0.7880
13-6-2007	6	14	0	20	0	0.0577
13-6-2007	4	13	3	20	15	0.0245
Total	26	42	15	80	18,75	P= 0.03406 *
Date	**CHA1144 *M.brassicae***	**CHA1144 *p.rapae***				
30-3-2007	6	8	6	20	30	0.3953
4-4-2007	5	12	3	20	15	0.0717
25-4-2007	6	10	4	20	20	0.2272
Total	17	30	13	60	21.67	P= 0.03947 *

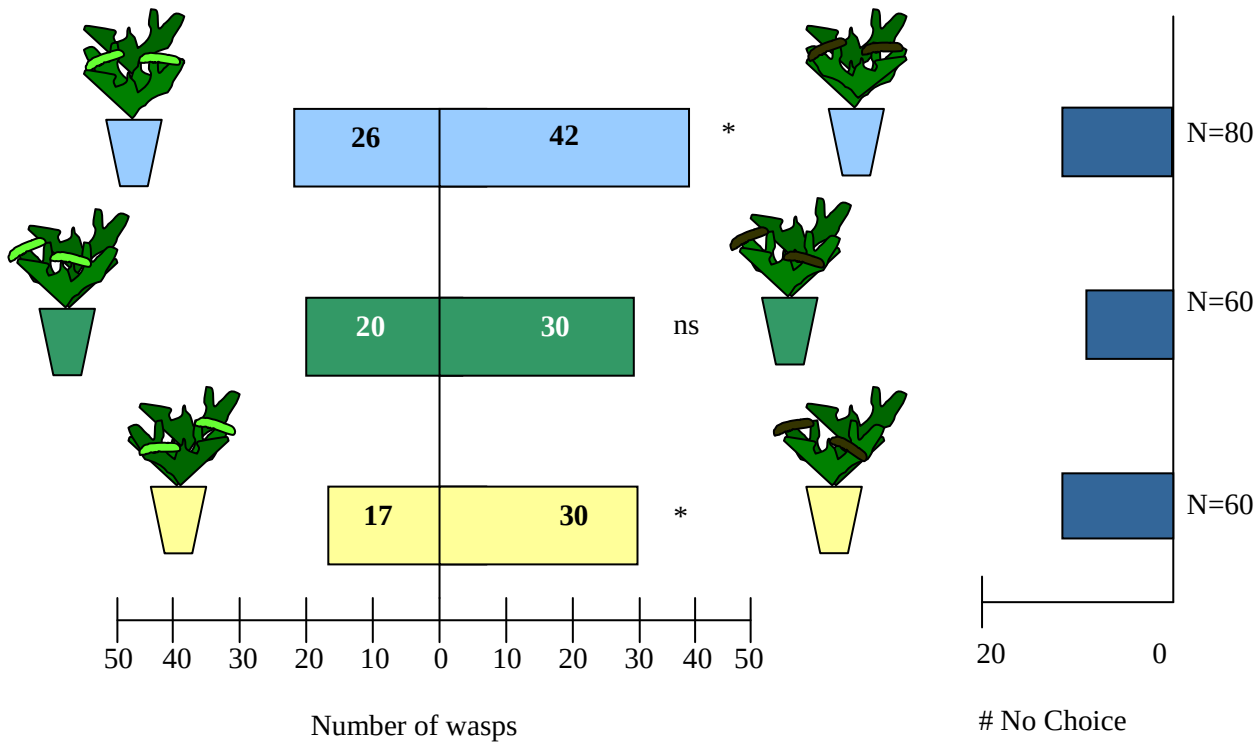

Figure 3.5. *D.semiclausum* response for different *Pf* treatments after 24h exposure of *M.brassicae* and *P.rapae* on *B.oleracea.* Control treatments and *Pf* strains CHA0 and CHA1144 treatments are indicated with light green, blue and yellow respectively. Bright green and dark green indicate the herbivores *M.brassicae* and *P.rapae*, respectively. Figures displayed in the columns define the total number of wasps that chose a specific treatment.

3.3 Gene expression by qRT-PCR

Five genes involved in different plant defence mechanisms were studied for their expression levels upon caterpillar attack in the presence of PGPR *Pf* strains CHA0 and CHA1144. *B. oleracea* gene expression levels were quantified by quantitative Real-Time PCR and Ct data was normalized with reference gene *GADPH*. Complete results of T-probabilities of pair wise differences of each specific primer are shown in **tables 3.16-3.20 (**appendix II). Error bars in graphs are based on SD values and do not include confidence intervals, therefore overlapping error bars can not be regarded to as insignificant.

3.3.1 Effects of PGPR on *BoPRI* transcript accumulations after caterpillar infestation

M. brassicae infestation on gene expression

Figure 3.6 displays the mean normalized expression levels of three replicates for gene *BoPR1* after zero and six hours of *M.brassicae* feeding. **Table 3.6** (appendix II) shows the T-probabilities of pair wise differences. Yellow compartments signify data with significant difference (P<0.05) between treatments. Systemic and local leaves were included. When comparing both the uninfested control that was unexposed to *M.brassicae* and the local leaf of the control exposed to *M.brassicae* to the systemic leaf of the control plant exposed to *M.brassicae*, there was a significant decrease in *PR1* expression for the systemic leaf. This down-regulation was not observed for CHA0 systemic leaves. There was no significant difference in *PR1* expression when comparing among control, *Pf* CHA0 *Pf* CHA1144 treatments for both the systemic and local leaves. *Pf* CHA1144 uninfested plants were excluded from data, due to high standard deviations.

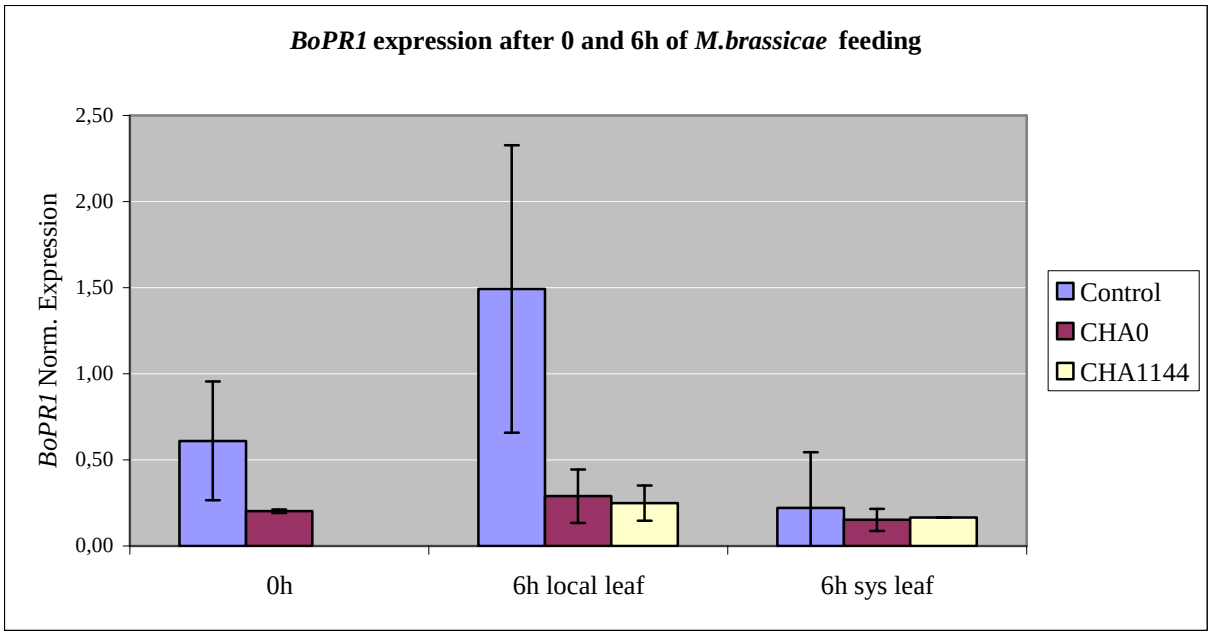

Figure 3.6. Transcript accumulations of the *B.oleracea* specific *PR1* after zero and six hours of *M.brassicae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

P. rapae infestation on gene expression

Figure 3.7 and **table 3.7** (appendix II) show normalized expression levels and T-probabilities of pair wise differences for *BoPR1* expression in plants exposed to *P.rapae*. An up-regulation of *PR1* was observed for the local leaves of the control plants compared to the systemic leaves of control plants (P=0.021). However, control plants unexposed to *P.rapae* did not significantly differ from either local and systemic leaves of the control treatment.

No difference was observed for *Pf* CHA0 and *Pf* CHA1144 treated plants when comparing between leaves of plants after 0h of *P.rapae* exposure, systemic and local leaves.
There was no significant difference in *PR1* expression between control, *Pf* CHA0 and *Pf* CHA1144 treated plants.

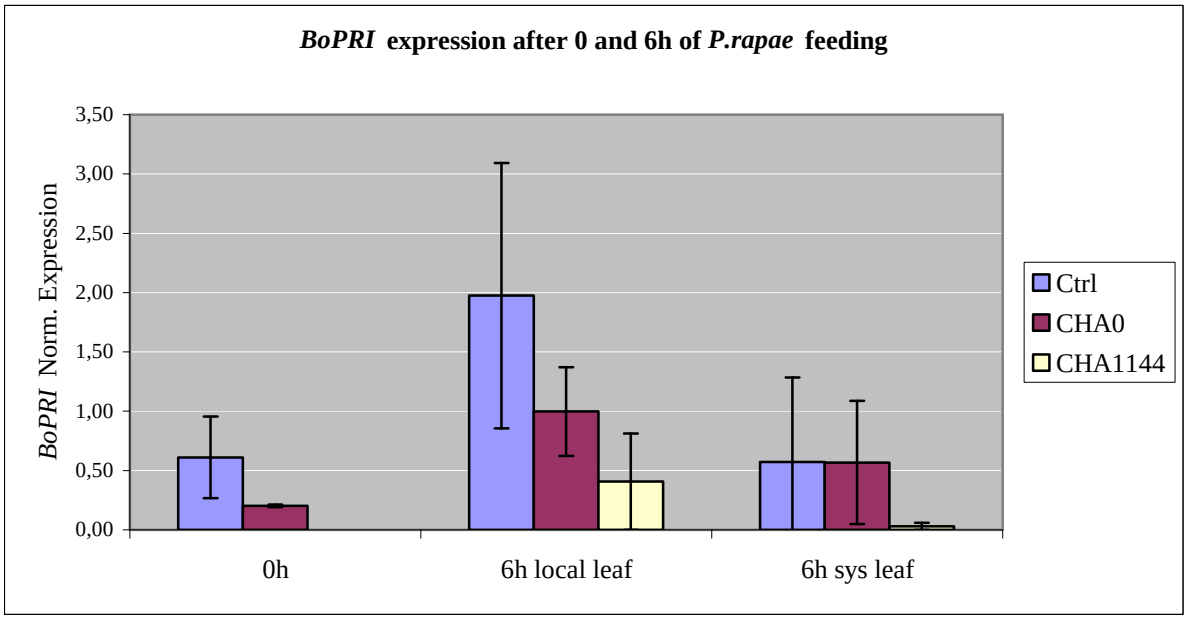

Figure 3.7. Transcript accumulations of the *B.oleracea* specific *PR1* after zero and six hours of *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

BoPR1 expression comparisons between *P. rapae* and *M. brassicae* infestation were presented in **figure 3.8**. No significant difference was found between *P.rapae* and *M.brassicae* damage when same *Pf* and leaf treatments were compared (P>0.05).

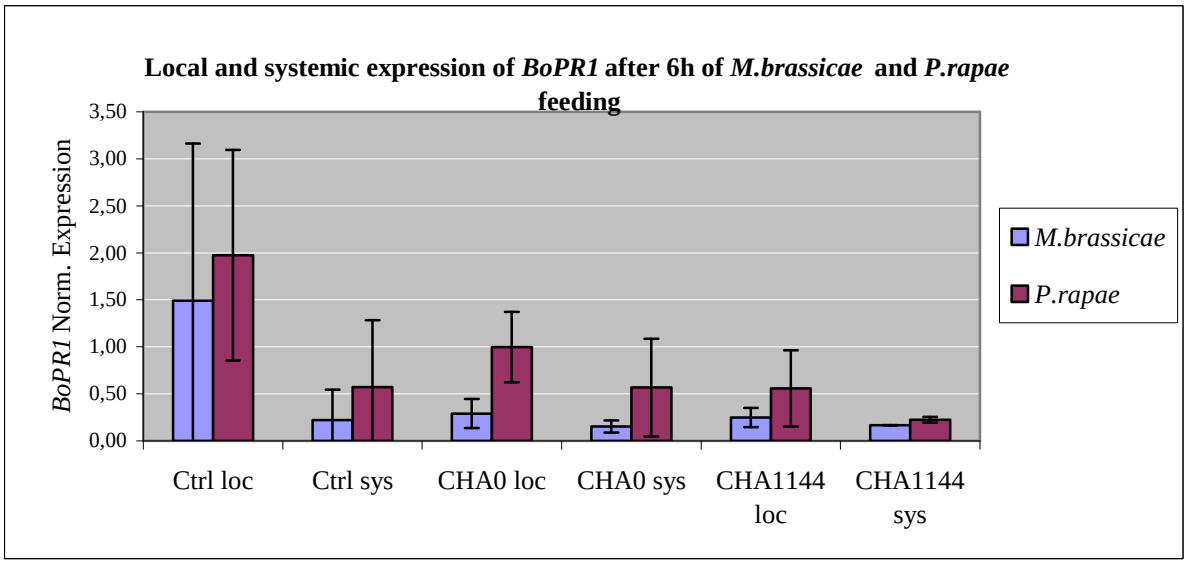

Figure 3.8. Transcript accumulations of the *B.oleracea* specific *PR1* after six hours of either *M.brassicae* or *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. No significant difference in *BoPR1* expression was seen when comparing between generalist and specialist caterpillar species. Sys indicates systemic.

3.3.2 Effects of PGPR on *BoDEF* transcript accumulations after caterpillar infestation

M. brassicae infestation on gene expression

Figure 3.9 and **table 3.8** (appendix II) show *BoDEF* normalized expression after feeding of the caterpillar *M.brassicae* for zero and six hours. When comparing control zero hours infested leaves to control six hours infested local and systemic leaves, a strong down-regulation was observed ($P<0.05$). No significant difference was found between *BoDEF* expression in local and systemic leaves of control plants ($P>0.05$). For undamaged *Pf* CHA0 infested plants, a significant difference was observed when compared to systemic leaves of damaged plants. No down-regulation was observed when undamaged CHA0 plants were compared to local leaves of damaged plants. There was no significant difference between local and systemic leaves of *Pf* CHA0 infested plants. For *Pf* stain CHA1144 no difference between undamaged and damaged leaves was observed, regarding to *BoDEF* expression levels. When comparing between control and *Pf* treatments for undamaged plants, CHA1144 inoculation induced a significant down-regulation in *BoDEF* expression ($P=0.026$) compared to the control plant. This was not observed when CHA0 was compared to the control and *Pf* CHA1144 treatments. No significant difference was found when looking at *BoDEF* expression for undamaged plants and caterpillar damaged plants for systemic and local leaves for the different treatments.

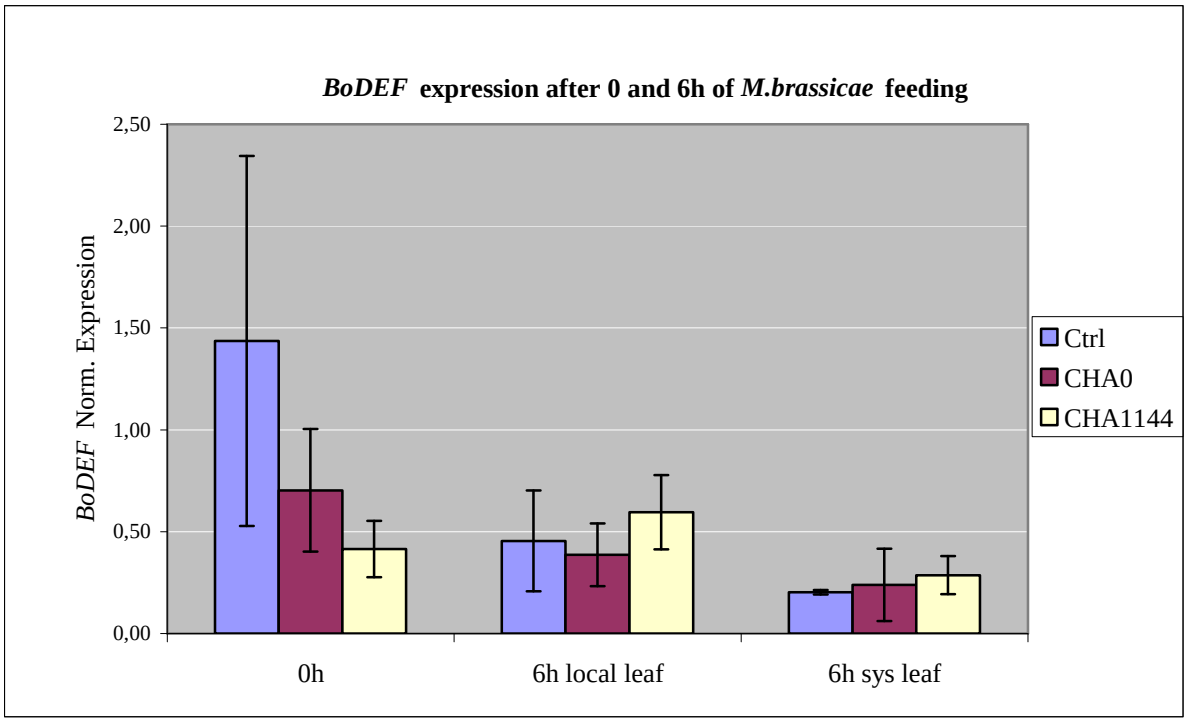

Figure 3.9. Transcript accumulations of the *B.oleracea* specific *DEF* gene after zero and six hours of *M.brassicae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

P. rapae infestation on gene expression

Figure 3.10 and **table 3.9** (appendix II) show *BoDEF* normalized expression levels after feeding of the caterpillar *P.rapae* for zero and six hours. Systemic leaves of the control treatment were significantly down-regulated compared to undamaged and locally damaged leaves of the control treatment, concerning *BoDEF* normalized expression. No difference was observed for the control between undamaged and locally damaged leaves. *BoDEF* was up-regulated in local leaves of *Pf* CHA0 treated plants when compared to both undamaged CHA0 treated plants and when compared to systemic leaves of *Pf* CHA0 treated plants. A highly significant up-regulation was observed for local leaves of *Pf* CHA1144 treated plants when compared to systemic leaves of *Pf* CHA1144 treated plants.

CHA1144 locally damaged leaves showed no difference in *BoDEF* expression when compared to the undamaged *Pf* CHA1144 treated plants. No difference was found when *Pf* CHA1144 systemic leaves were compared to leaves of undamaged CHA1144 infested plants.
When comparing between *Pf* treatments, it was observed that *Pf* CHA1144 treated undamaged plants differ significantly from undamaged control plants. This difference was not found for *Pf* CHA0 treated undamaged plants compared to both *Pf* CHA1144 treated undamaged plants and undamaged control plants. No significant difference was found for comparisons between systemic leaves of different *Pf* treatments. Also no significant difference was found when local leaves between the different *Pf* treatments were compared.

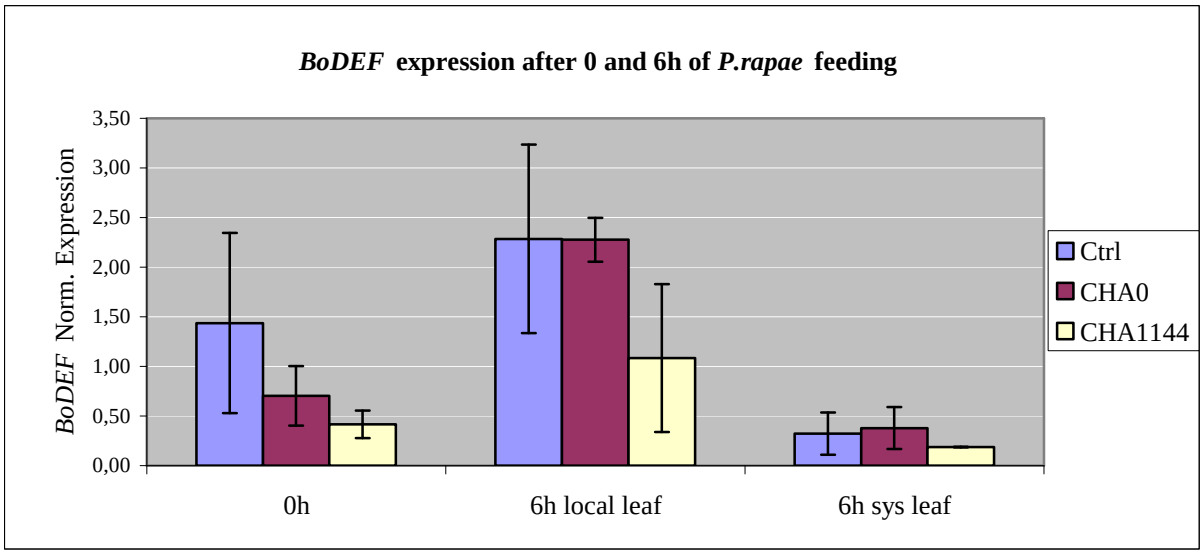

Figure 3.10. Transcript accumulations of the *B.oleracea* specific *DEF* gene after zero and six hours of *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

BoDEF expression comparisons between *P. rapae* and *M. brassicae* infestation were presented in **figure 3.11**. In both local leaves of control and *Pf* CHA0 treated plants *BoDEF* transcript accumulations were significantly induced when damaged by *P. rapae*. The other treatments showed no significant difference between caterpillar damage.

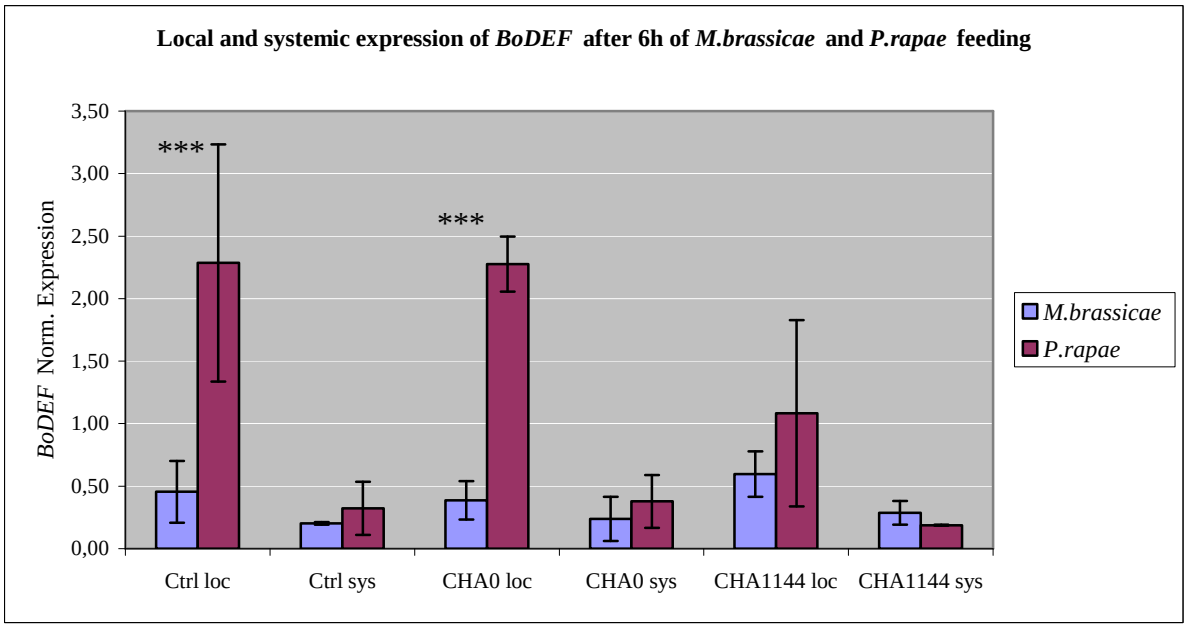

Figure 3.11. Transcript accumulations of the *B.oleracea* specific *DEF* gene after six hours of either *M.brassicae* or *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys and loc indicate systemic and local, respectively.

3.3.3 Effects of PGPR on *BoLOX* transcript accumulations after caterpillar infestation

M. brassicae infestation on gene expression

Figure 3.12 displays *BoLOX* expression levels after zero and six hours of *M. brassicae* feeding. *BoLOX* expression in local leaves of control treated plants was significantly up-regulated (**table 3.10**; appendix II) when compared to systemic leaves of control plants and leaves of undamaged control plants. For CHA0 treated plants *BoLOX* was up-regulated in both local and systemic leaves compared to control leaves of undamaged plants. Additionally, local leaves were up-regulated upon *M. brassicae* when compared to systemic leaves (P=0.017). Plants treated with *Pf* strain CHA1144 showed a highly significantly up-regulation of *BoLOX* in local leaves compared to undamaged control leaves (P<0.001). No differences were found in *BoLOX* expression between systemic leaves and CHA1144 0h treated plants and local leaves.

There was no difference found in gene expression between the undamaged controls for the different *Pf* treatments. Concerning systemic leaves, a significant increase in gene expression was found when comparing between control and CHA0 treated plants (P=0.048). No significant difference was found in *BoLOX* expression when comparing between local leaves for the different *Pf* treatments.

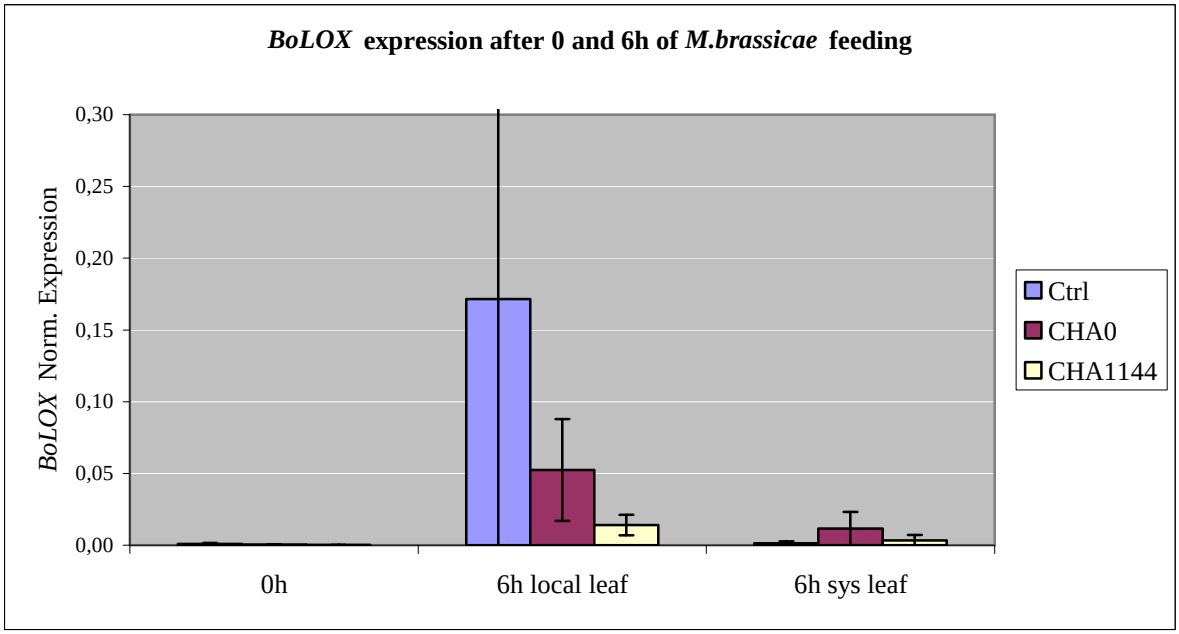

Figure 3.12. Transcript accumulations of the *B.oleracea* specific *LOX* gene after zero and six hours of *M.brassicae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

P. rapae infestation on gene expression

Figure 3.13 and **table 3.11** (appendix II) show normalized expression levels and T-probabilities of pairwise differences for *BoLOX* after *P. rapae* damage. When undamaged control plants and systemic leaves of *P. rapae* damaged plants are compared to local leaves of control plants, a strong up-regulation in *BoLOX* expression was observed in the local leaf. *Pf* CHA0 and *Pf* CHA1144 infested plants showed a similar up-regulation of *BoLOX* expression in the local leaf. In addition, *Pf* CHA1144 infested plants showed a significant increase in *BoLOX* expression when undamaged leaves and systemic leaves were compared. When comparing between control, *Pf* CHA0 and *Pf* CHA1144 treated plants, no significant difference in normalized expression of *BoLOX* was found for as well leaves unexposed to *P.rapae* as systemic leaves and damaged local leaves of *B.oleracea.*

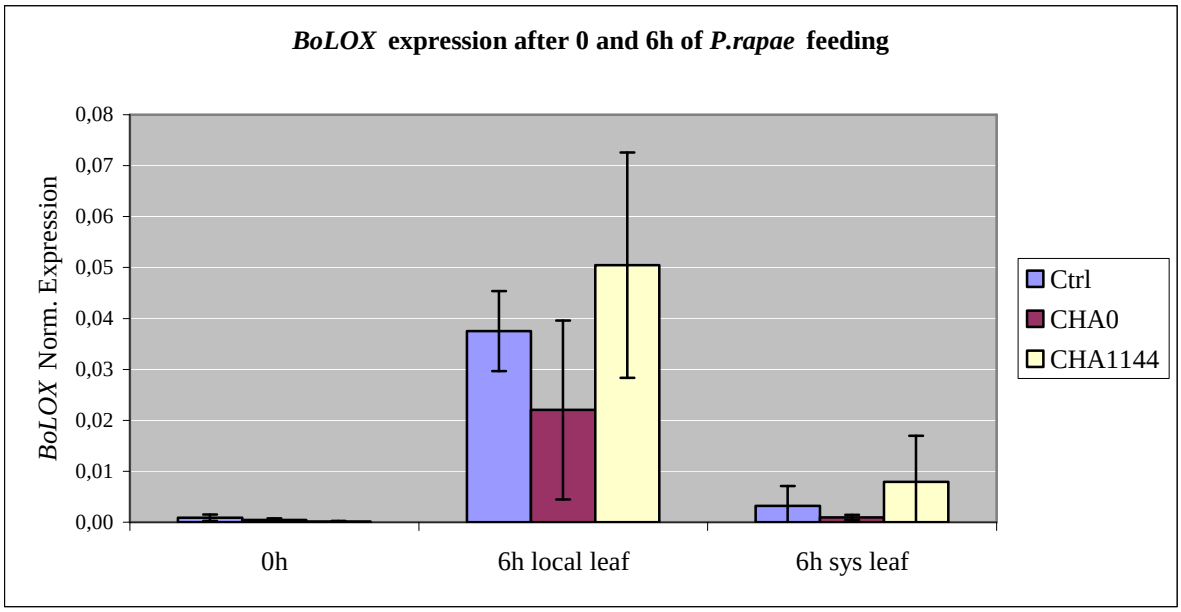

Figure 3.13. Transcript accumulations of the *B.oleracea* specific *LOX* gene after zero and six hours of *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

BoLOX expression comparisons between *P. rapae* and *M. brassicae* infestation were presented in **figure 3.14**. When comparing systemic leaves of *Pf* CHA0 infested plants, *BoLOX* transcription was significantly induced upon *M. brassicae* feeding (P=0.036) (**table 3.18;** appendix II). No significant differences between caterpillar damage were found for other treatments.

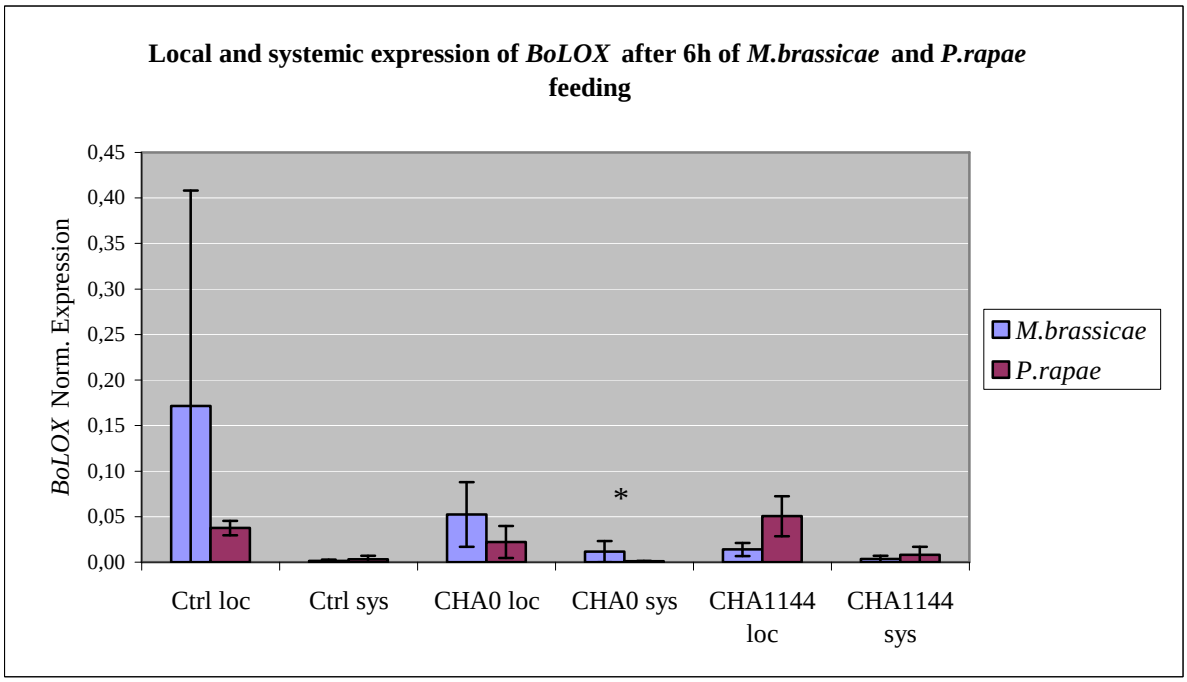

Figure 3.14. Transcript accumulations of the *B.oleracea* specific *LOX* gene after six hours of either *M.brassicae* or *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys and loc indicate systemic and local, respectively.

3.3.4 Effects of PGPR on *BoMYC* transcript accumulations after caterpillar infestation

M. brassicae infestation on gene expression

Figure 3.15 displays normalized expression for *BoMYC* in leaves exposed to *M. brassicae.* T-probabilities of pairwise differences (**table 3.12;** appendix II**)** show that *BoMYC* expression in local leaves of control plants was significantly up-regulated compared to systemic and undamaged control leaves. No significant difference in *BoMYC* expression was observed between systemic and undamaged control leaves. For *Pf* CHA0 infested plants, *BoMYC* was up-regulated in both systemic and local leaves compared to the control leaf. No significant difference in gene expression was observed between systemic and local leaves damaged by *M. brassicae*. For *Pf* CHA1144 infested plants, a highly significant up-regulation in local leaf gene expression was observed, compared to the control. A difference was also observed for *Pf* CHA1144 when 0h infested plants were compared to systemic leaves of 6h infested plants. However, no significant difference was observed CHA1144 local leaf when compared to CHA1144 systemic leaf. No significant difference was observed for *BoMYC* expression between control, *Pf* CHA0 and *Pf* CHA1144 treated plants when comparing between treatments.

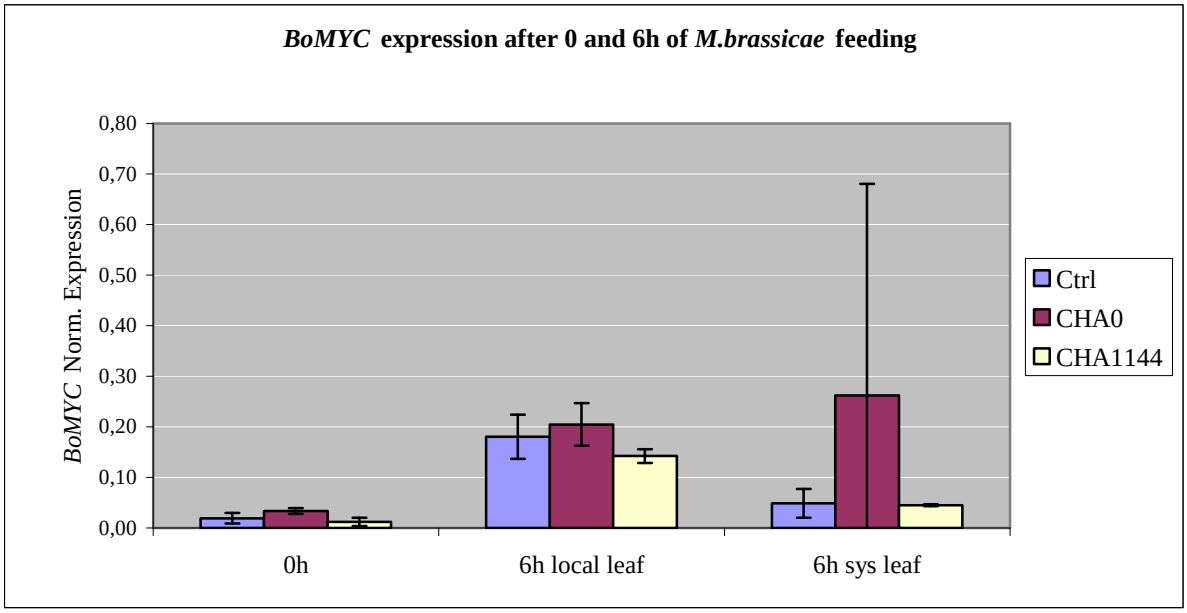

Figure 3.15. Transcript accumulations of the *B.oleracea* specific *MYC* gene after zero and six hours of *M.brassicae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

P. rapae infestation on gene expression

Normalized expression levels and T-probabilities of pair wise differences *BoMYC* after *P. rapae* feeding were shown in **figure 3.15** and **table 3.13** (appendix II). A strongly significant increase in gene expression was observed in local leaves of control treated plants compared to systemic and undamaged control leaves. No difference was observed between systemic leaves and leaves unexposed to *P.rapae* of the control treatment.

Similar results were also observed for *Pf* infested CHA0 and CHA1144 plants. Complementary, in *Pf* infested CHA1144 plants there was an up-regulation seen of *BoMYC* expression in systemic leaves when compared to leaves unexposed to *P. rapae*. When comparing between different soil treatments, a significant difference (P=0.026) was shown for plants unexposed (0h) to *P.rapae* and *Pf* CHA0 treated plants compared to *Pf* CHA1144 treated plants. No significant difference in *BoMYC* expression was perceived between systemic and local leaves of the different treatments.

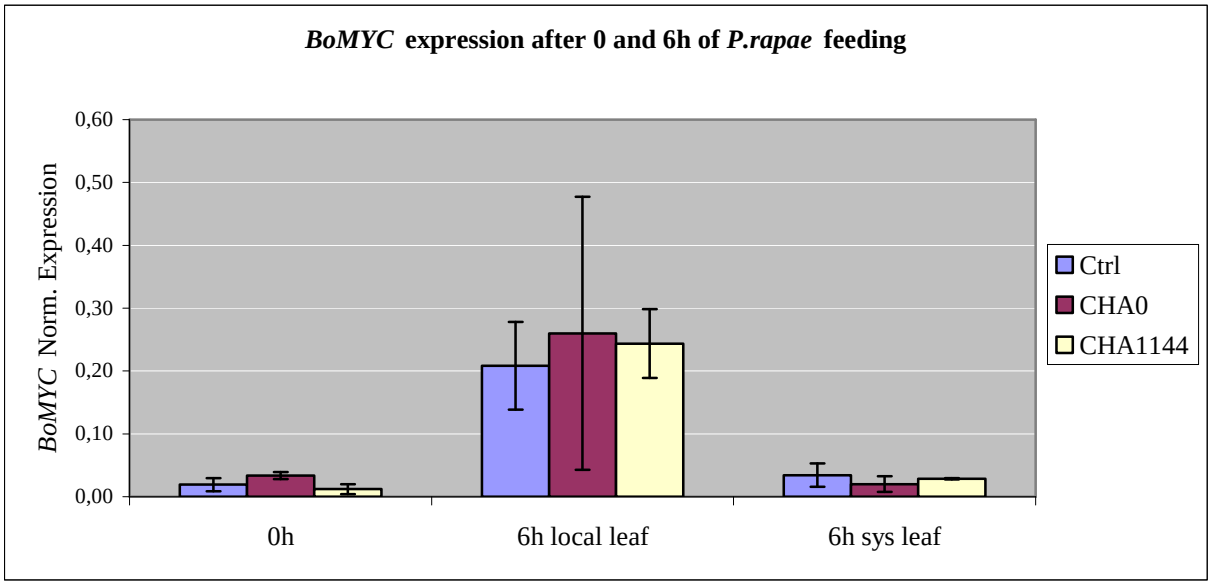

Figure 3.16. Transcript accumulations of the *B.oleracea* specific *MYC* gene after zero and six hours of *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

BoMYC expression comparisons between *P. rapae* and *M. brassicae* infestation were presented in **figure 3.17**. For plants infested by *Pf* CHA0, a strong down-regulation of *BoMYC* was observed in systemic leaves for *P.rapae* damaged plants compared to *M.brassicae* damaged plants. No significant effect between caterpillar damage was observed for the other treatments.

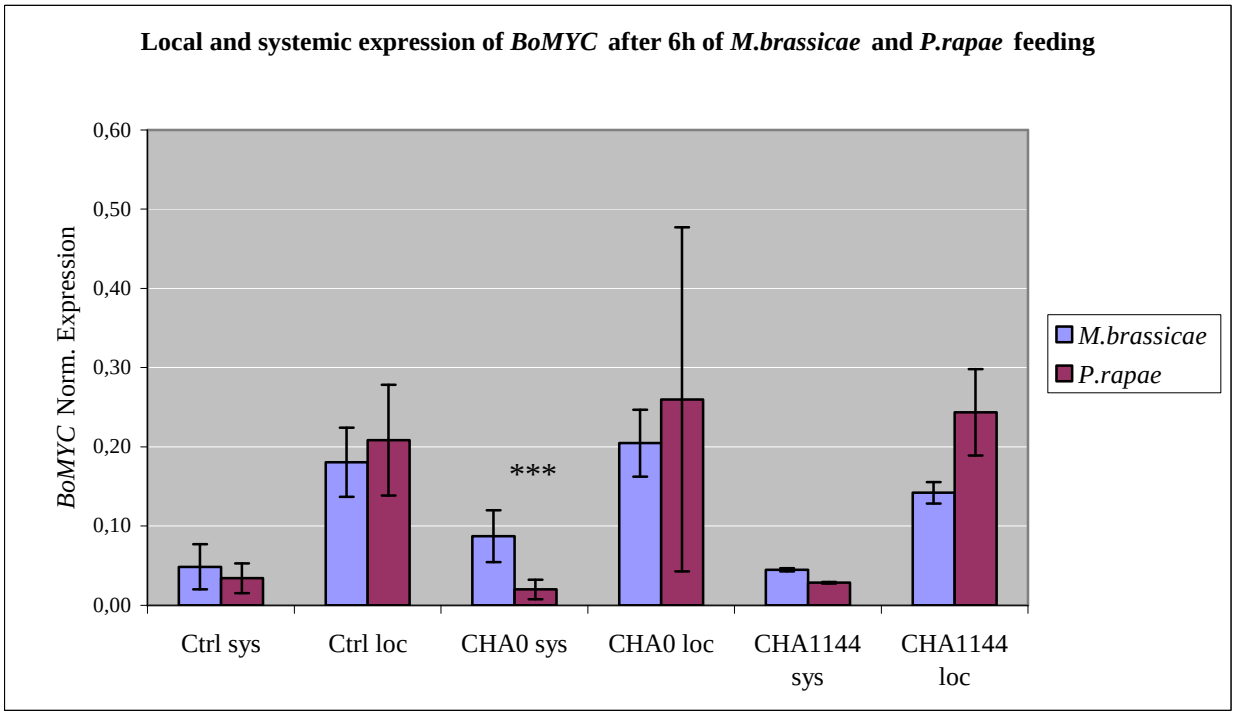

Figure 3.17. Transcript accumulations of the *B.oleracea* specific *MYC* gene after six hours of either *M.brassicae* or *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys and loc indicate systemic and local, respectively.

3.3.5 Effects of PGPR on *BoMYR* transcript accumulations after caterpillar infestation

M. brassicae infestation on gene expression

Figure 3.18 and **table 3.14** (appendix II) show the results of normalized expression levels and T-probabilities of pair wise differences for *BoMYR* after *M.brassicae* feeding on plants treated with either MgSO4, *Pf* CHA0 or transgenic *Pf* CHA1144. **Table 3.14** (appendix II) indicates that normalized expression of *BoMYR* did not significantly differ between different soil treatments. No significant changes were observed when undamaged leaves were compared to local and systemic leaves per treatment and no significant difference was observed between systemic and local leaves per treatment. Data for *BoMYR* normalized expression in local leaves after MgSO4 soil treatment were excluded from the results, because expression data and standard deviations were extremely high.

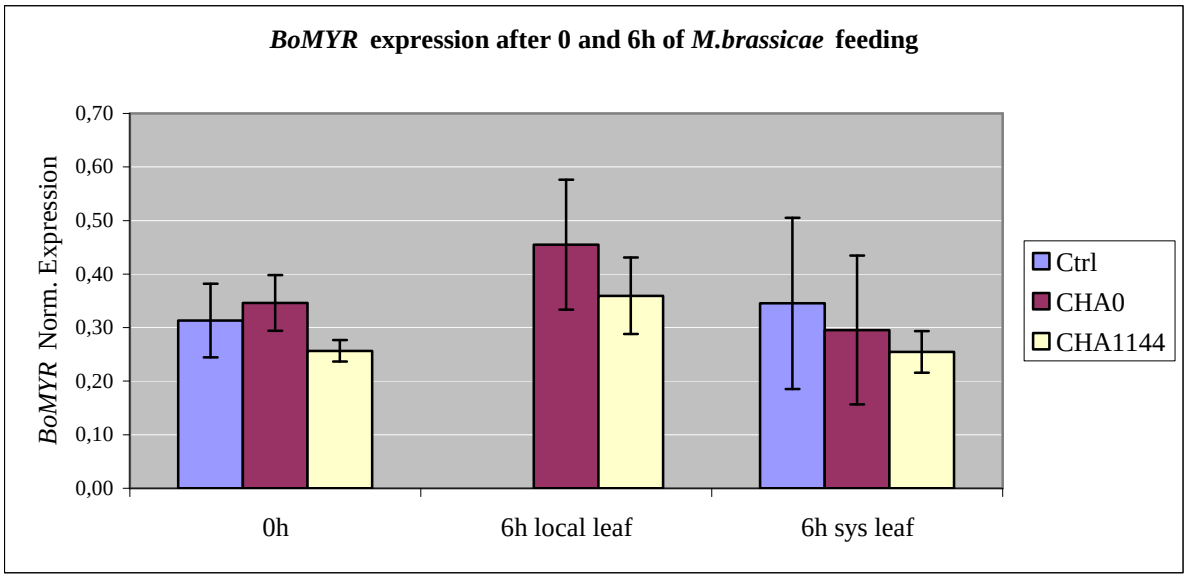

Figure 3.18. Transcript accumulations of the *B.oleracea* specific *MYR* gene after zero and six hours of *M.brassicae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

P. rapae infestation on gene expression

No difference was observed for *BoMYR* expression after zero and six hours of *P. rapae* feeding on control plants for local as well as systemic leaves from **figure 3.19** and **table 3.15** (appendix II). In local leaves of *Pf* CHA0 infested plants, a significant up-regulation in *BoMYR* expression levels was observed when compared to *BoMYR* expression in systemic leaves of Pf *CHA0* infested plants. This difference was not observed when local leaves were compared to leaves unexposed to *P. rapae* and there was no difference in gene expression of *BoMYR* between the systemic leaf and the control leaf of *Pf* CHA0 treated plants. For *Pf CHA1144*, there was no alteration in gene expression detected among systemic leaves, local leaves and leaves unexposed to *P.rapae*. There were no differences in *BoMYR* expression found when comparing between different soil treatments.

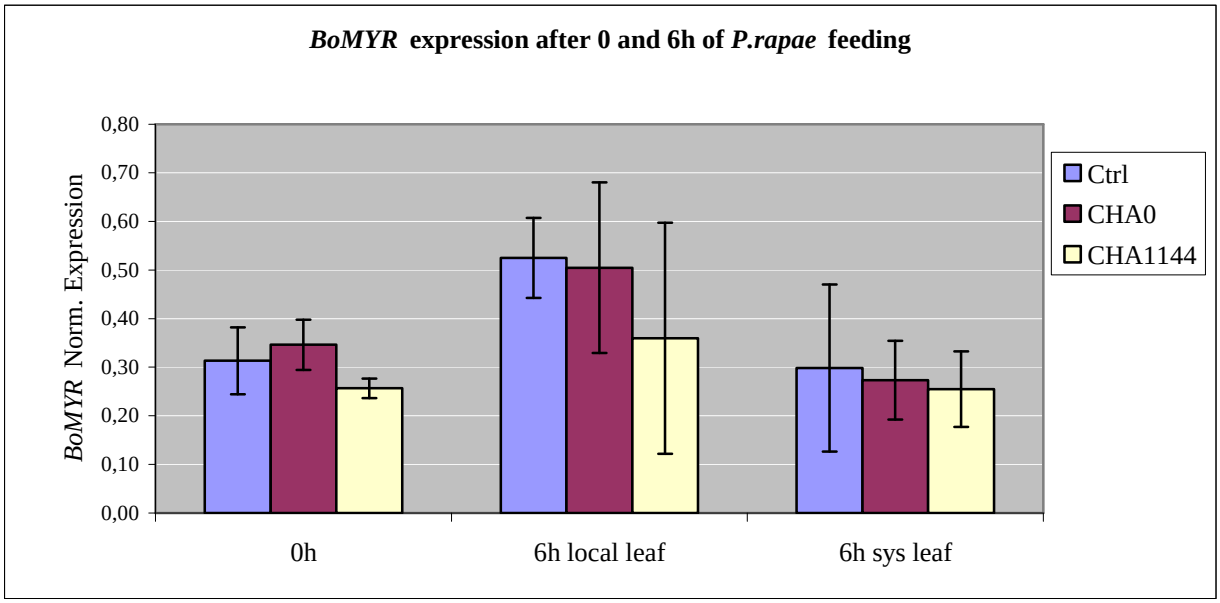

Figure 3.19. Transcript accumulations of the *B.oleracea* specific *MYR* gene after zero and six hours of *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys indicates systemic.

BoMYR expression comparisons between *P. rapae* and *M. brassicae* infestation are presented in **figure 3.20** and **table 3.20** (appendix II). It was found that leaves exposed to different *Pf* treatments did not significantly differ in *BoMYR* expression levels upon leaf damage elicited by either *P.rapae* or *M.brassicae*.

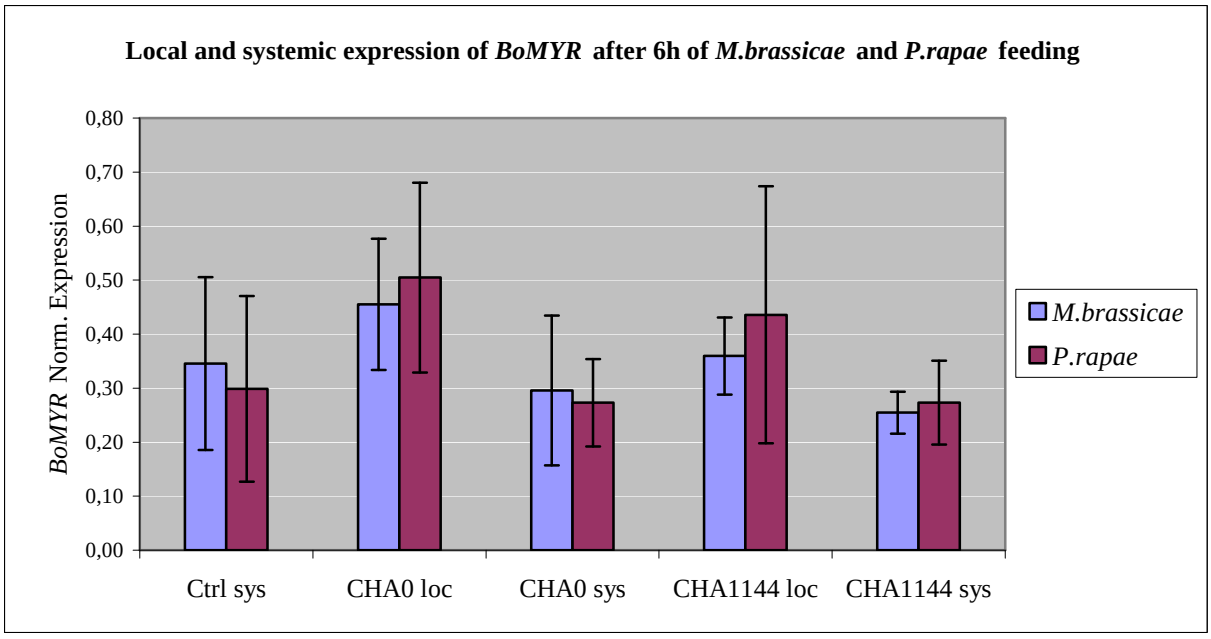

Figure 3.20. Transcript accumulations of the *B.oleracea* specific *MYR* gene after six hours of either *M.brassicae* or *P.rapae* feeding. Bars indicate the normalized expression mean (±SD) of three biological replicates for the different *Pf* treatments. Sys and loc indicate systemic and local, respectively.

Discussion

Extensive research has demonstrated that plants are capable of defending themselves directly against herbivorous insects by producing toxins or repellents or indirectly by attracting natural enemies of these plant predators in a tritrophic interaction (Van Poecke and Dicke., 2004). Plant defence against herbivorous attackers is mediated by three phytohormones, JA, ET and SA that play a major role in partially overlapping signalling pathways (Van Poecke and Dicke., 2004, Pieterse et al., 1998, Pieterse et al., 1996, Maurhofer et al., 1994). Previously it was demonstrated that several *Pseudomonas fluorescens* strains in the soil are capable of 'priming' plants, resulting in a faster, stronger or both expression of genes, involved in plant defence signalling pathways, upon pathogen attack (Conrath et al., 2006, Siddiqui et al., 2005; Haas and Keel., 2003; Laville et al.,1992, Leon-Kloosterziel et al., 2005; Pieterse et al.,1997; Pieterse et al., 1996; van Wees et al., 1997). More recently, tetratrophic interactions involving non-pathogenic soil bacteria, host plants, herbivorous insects and parasitoid wasps, have been studied for their effect on plant defence (Van Oosten et al. 2007, Zheng et al., unpublished results). In this study we combined ecological and molecular tools to examine the effect of two *Pf* strains, CHA0, its derivative mutant strain CHA1144 (transgenic mutant of *Pf* CHA0) and WCS417r on direct and indirect plant defence as a response to *P. rapae* and *M. brassicae* feeding.

Effects of Pf strains on direct plant defence

It was previously demonstrated that *Pf* strain WCS417r negatively effected the larval growth of generalist feeder *M.brassicae* on *B. oleracea* plants. In contrast, induced plants defences by *Pf* WCS417r displayed no effect on the weight gain of specialist feeder *P.rapae* (Zheng et al., unpublished results). This is partially inconsistent with my observations that WCS417r did not alter larval body weight of *M. brassicae* on *B. oleracea*, compared to control plants (**table 3.1**). This inconsistency can be explained by various experimental factors that influence the outcome. Experiments were conducted during different time points, which might have had an influence on bacterial cultures, plant growth and insect performance. Furthermore, infested larval age and size, greenhouse conditions and seedling conditions might have unintentionally differed.
For *P. rapae* we find the results for *Pf* WCS417r (**table 3.2**) to be in consensus with previous findings (Van Oosten et al., 2007; Zheng et al., unpublished results). Van Oosten did not observe a significant difference in the average weight of *P. rapae* larvae grown on *A.thaliana* in the presence of WCS417r compared to ISR uninduced plants.
To a limited extent we observed a insignificantly lower larval body weight for both herbivorous insects on WCS417r infested plants, when compared to control plants (**figure 3.1** and **figure 3.2**).
For *Pf* wild type strain CHA0, we observed no difference in the larval body weight of generalist feeder *M.brassicae*, indicating that larvae were unaffected in their feeding behaviour by induced plant defences (**table 3.1**).
For *P.rapae* larvae residing on *Pf* CHA0 induced plants a highly significant reduction (P=0.002) in average larval growth was perceived when compared to control plants (**table 3.2**). The effect of CHA0 on direct plant defense against caterpillar damage has not been investigated before. Previous studies with plant pathogens such as, *F.oxysporum, T. basicola, P.ultimum*, demonstrated that plants showed a reduction in disease symptoms when grown in the presence of *Pf* CHA0 (Pechy-Tarr et al., 2005; Naseby et al., 2001; Laville et al., 1992). The reduction in larval body weight indicates that CHA0, as a biocontrol strain against fungal and bacterial plant pathogens, might be a potential biological control agent against specific insect pests in the future.
No significant effect on the average body weight was observed for generalist feeder *M. brassicae* larvae residing on *Pf* CHA0 transgenic mutant strain CHA1144 infested plants, when compared to larvae remaining on uninfested plants (**table 3.1**). This indicates that *Pf* CHA1144 infested plants do not differ from control plants in direct defense against *M. brassicae* larvae.
Average weight gain of *P.rapae* larvae was highly significantly reduced on CHA1144 plants compared to control and WCS417r plants (P<0.001 and P=0.004, respectively) (**table 3.2**).

These results contradict the hypothesis that *Pf* CHA1144 is impaired in the induction of ISR due to a triple mutation in the GacS/GacA system, causing *Pf* CHA1144 to be impeded in DAPG production (Kay et al., 2005; Reimmann et al., 2005; Haas and Keel, 2003; Iavicoli et al., 2003). During bacterial growth on KB nutrient plates it was viewed that strain CHA1144 grew faster compared to the wild type strains, however, this was not quantified. In contrast, previous experiments showed that *in vitro* growth of strain CHA1144 was indistinguishable from that of the wild type CHA0 (Kay et al., 2005).
An explanation for these results is that the transgenic mutant compensates its deficiencies by addressing other genetic properties, resulting in ISR. Additional small RNAs with different functions might participate in the activation of exoproducts synthesis via the GacS/GacA system (Kay et al., 2005). This was not observed when experiments concerning the role of *Pf* CHA1144 in ISR were conducted, where it was perceived that pathogens were more virulent on *Pf* CHA1144 infested plants compared to wild type *Pf* strain CHA0 (Kay et al., 2005). Further research for physiological properties of the transgenic strain might provide a better inside in *Pf* CHA1144 performance.
After eight days of *M. brassicae* feeding all larvae were found again on *B.oleracea*. This was remarkable since the transfer of *M.brassicae*, in contrast to the transfer of *P.rapae* larvae, is difficult and larvae do not stick to the plants and easily fall of. It appeared that there was less movement of *M. brassicae* larvae compared to *P. rapae* on plants, while *P. rapae* had been roaming around, as larval quantities differed from initial quantities per plant after eight days of feeding. Dynamic larval movement might indicate that *P. rapae* larvae were repelled by the plant and that *B. oleracea* utilizes induced defence against specialist *P. rapae* then against generalist *M. brassicae*. A hypothesis that supports this outcome, is that plants are able to recognize specialist herbivores better, because they are more frequently present on the plant, resulting in stronger and more diverse defense gene expression then when a plant encounters an infrequent generalist attacker (Reymond et al., 2004). In contradistinction, it is regularly observed that specialist herbivores more easily overcome plant defence responses (Reymond et al., 2004).
From a biological point of view, larval feeding experiments might have been improved by direct oviposition of eggs on ISR induced and uninduced plants, instead of transferring of neonate larvae to plants, as this reflects a natural situation and does not affect larval fitness. Innovative techniques, to acquire equal emergence of the larvae and to restrict the number of eggs that are deposited on the plant, might provide us with tools to improve future experimental approaches.

Effects of Pf strains on indirect plant defence

To determine the effect of PGPR *Pf* strains CHA0 and CHA1144 (ΔrsmXYZ) on indirect plant defence, we looked at the preference of parasitoid wasp *D.semiclausum* for the different plant treatments. Previous research on model plant *A. thaliana* showed that the presence of *Pf* WCS417r in the soil did not change the attractiveness of the plants to *C. rubecula* upon feeding of *P. rapae* in the wind tunnel (Van Oosten et al., 2007). Similar results were confirmed by Zheng et al. (unpublished results) with *C. glomerata* on *B. oleracea.* However, in the same study it was shown that there was a highly significant preference of *D. semiclausum* for *B. oleracea* plants infested with *Pf* WCS417r when compared to uninfested plants in the Y-tube experiments (Zheng et al., unpublished results).
Here, we found that plants treated with *Pf* transgenic mutant strain CHA1144 were highly significantly preferred by the parasitoid wasp ($P<0.001$) over control plants in a Y-tube experimental set-up (**figure 3.3**). This preference for plants grown in the presence of *Pf* CHA1144 was not observed when comparing between *Pf* CHA0 and *Pf* CHA1144 infested plants after *P.rapae* exposure, nevertheless a tendency toward plants infested by the transgenic line was observed. In addition, plants exposed to *M.brassicae* are significantly more attractive to *D. semiclausum* when infested with *Pf* CHA11144 compared to *Pf* CHA0 ($P<0.005$)(**table 3.4.**). Preference for plants grown in the presence of *Pf* CHA1144 can be explained by a modification in the production of secondary metabolic products (Kay et al., 2005; Reimmann et al., 2005; Haas and Keel, 2003; Iavicoli et al., 2003) resulting in a changed volatile pattern excreted by the soil *Pf* bacteria. A difference in the volatile expression pattern in the soil might result in a change in *D. semiclausum* preference for a specific treatment. Both plants infested by *Pf* CHA0 and control plants were similar attractive for parasitoid *D. semiclausum* (**table 3.3** and **table 3.4**) after either *M.brassicae* or *P.rapae* exposure.

An interpretation could be that the wasp could not distinct between volatiles excreted by these different treated plants, which might point out that volatiles excreted from either soil or the plant or both, are less distinctive than the volatiles produced by the mutant strain. Trapped volatile analyses might reveal more inside about this. It was perceived, but not quantified, during the culturing of *Pf* CHA1144, that this strain excreted a strong penetrating smell compared to the wild type strain. This might explain why CHA1144 infested plants were preferred by *D.semiclausum* over CHA0 infested plants exposed to *M. brassicae* (**table 3.4**).
For *P.rapae* it was shown that *D. semiclausum* wasps prefer plants infested by the mutant strain over plants infested by the wild type strain. A similar pattern was observed for *M. brassicae* infested plants, although not significant (**figure 3.4**). As observed in the direct defence experiment, this can be explained by a hypothesis that plants might recognize specialist herbivores better and therefore are capable of inducing stronger and more specific defense gene expression against specialist *P.rapae* compared to generalist *M. brassicae* (Reymond et al. 2004). Another explanation might be the divergent multiple abiotic and biotic factors during the experimental set-up that resulted in an insignificant outcome for the *M.brassicae* comparison between the mutant and control treated plants, since two out of three test days showed a strong tendency towards *Pf* CHA1144.
Recent findings showed a shift in parasitoid herbivore preference upon *Pf* WCS417r infestation between plants exposed to *P. rapae* and *M. brassicae* compared to control treatments. *D. semiclausum* wasps preferred *P. rapae* in uninfested plants and could not discriminate between both herbivorous insects on primed plants (Zheng et al., unpublished results). Our results show a similar shift in parasitoid preference when observing control and *Pf* CHA0 infested plants (**table 3.5**). For the transgenic line, *D. semiclausum* displays a preference for the specialist feeder *P. rapae*, similar to the control plant (**table 3.5**). Upon different *Pf* strains treated cabbage plants, different volatile patterns could be excreted after herbivous infestation. Trapped volatile GC-MS analyses are further needed to elucidate the outcome of these findings.

PGPR primed gene expression in B.oleracea

Plants can be primed by the presence of PGPR in the soil (Conrath et al., 2006; Loon et al., 1998). This phenomenon is also observed in plants that are 'primed' by volatile organic compounds excreted by neighbouring plants. In principle, a similar mechanism is exploited by the plants upon PGPR infestation, were the production of secondary metabolites is the crucial promoter for ISR (Kay et al., 2005; Reimmann et al., 2005; Haas and Keel, 2003). Priming by volatile organic compounds was observed to occur only in early stages of volatile perception (Ton et al. 2007). Here, we display the results of quantitative Real-Time PCR experiments after zero and six hours of *P. rapae* and *M. brassicae* exposure on control, *Pf* CHA0 and *Pf* CHA1144 treated plants.

Transcript profiles for pathogenesis-related *PR1* gene show decreased normalized expression levels upon *M. brassicae* feeding in systemic leaves compared to local and 0h exposed leaves (**figure 3.6**). This effect is not observed in both *Pf* CHA0 and *Pf* CHA1144 ($P>0.05$). In contrast, an accumulation in local and systemic leaves was expected. In earlier research, *M. brassicae* showed an accumulation of mRNA in local leaves (Zheng, unpublished results). Presumably, small significant changes between control, *Pf* CHA0 and *Pf* CHA1144 ($P=0.041$ and $P=0.042$) were caused by procedural defects and high SD.
P. rapae damage resulted in a higher local expression in 6h control plants compared to systemic expression in control plants (**figure 3.7**), which is caused by induced plant defence in local leaves.
For *PR1* we did not find the effect of priming in *Pf* CHA0 and also not in the mutant strain. This might indicate that *Pf* CHA0 is not capable of priming of *PR1* gene. In contrast, a gathering in pathogen-related gene expression was earlier demonstrated by Maurhofer et al. (1994), who found an accumulation of *PR1* in tobacco when induced by PGPR CHA0. A second conceivable explanation could be that six hours of caterpillar infestation is not sufficient to obtain enhanced defence in plants. This might also explain why expected resistance in local and systemic leaves, which was observed by of the control plant, fails to appear. In previous tests for insect induced resistance and the effect of ISR on plant resistance, plants were exposed to their attackers for a 24h (Van Oosten et al., 2007) and 48h time period (Zheng, unpublished results).

Implementation of a six hour insect exposure in this research was based on a finding of Ton et al. (2007), who purposed that priming by VOCs takes place during early stages of volatile perception.
Weak responses in *PR1* might indicate that the SAR pathway is not involved in priming displayed by the PGPR tested.

For the *B. oleracea* specific isolated *DEF* gene, down-regulation of expression was observed for systemic and local leafs after *M.brassicae* feeding compared to unexposed leafs of the control plant (**figure 3.9**). Also expression in systemic leaves of 6h CHA0 was lower than the expression of *BoDEF* in the undamaged plant. Presumable, this can be explained by the fact that low levels of defensin gene throughout the whole plant, that are constitutively expressed, are down-regulated upon attack to able the plant to invest more in local defence, however a up-regulation in local leaf expression of *BoDEF* was not observed. Reymond et al. (2004) observed no differences in expression *A. thaliana* plant defensin gene *PDF1.2* after *P. rapae* and *S. exigua* feeding in a micro-array expression analyses. Furthermore, Van Oosten et al. (2007) saw no difference in *PDF1.2* expression after *P.rapae* feeding in the presence of WCS417r. Expression of *BoDEF* might also not be induced after generalist *M.brassicae* feeding. In contrast, normalized expression levels after specialist *P.rapae* exposure show an expected higher expression level in local leaves compared to undamaged and systemic leaves for *Pf* CHA0 (**figure 3.10**). Local leaf mRNA levels also were significantly higher in control and CHA1144 treated plants. Which corresponds with the theory of induced defence upon caterpillar attack (Reymond et al. 2002, Poecke and Dicke, 2002).
For both *P. rapae* and *M. brassicae* CHA1144 undamaged plants compared to the control we find a down-regulation in *BoDEF* expression levels for the mutant strain after zero hours of insect feeding (**figure 3.9** and **figure 3.10)**. Mutant strain *Pf* CHA1144 is impaired in the production of a range of metabolites (Kay et al. 2005), of which DAPG has an important function in ISR induction. For the 0h exposed plants to caterpillar damage this result is inexplicable. Procedural failures might cause this unexpected outcome. Priming was not observed for *BoDEF*, which might indicate that *Pf* CHA0 is not capable of priming this gene or that duration of insect exposure was inadequate. If CHA0 is not capable of priming the *BoDEF* gene, it can be concluded that CHA0 does not alter the octadecanoid pathway in plants. Zheng (unpublished results) also observed no effect of *BoDEF* priming for PGPR WCS417r in plants exposed to *M. brassicae* and *P. rapae*.
However, when comparing normalized expression levels between both herbivorous larvae *P. rapae* and *M. brassicae* for the different treatments (**figure 3.11**), we observed a significantly higher expression of *BoDEF* in both local leaves after *P. rapae* exposure. Which can be explained by the fact that plants are better able to recognize specialist feeders (Reymond et al., 2004). There were no differences in gene expression between the herbivorous insects observed for local leaves *Pf* CHA1144 plants. Assumable, discrimination of herbivorous predation is less prevalent in the presence of *P.f.* CHA1144.

For *BoLOX* normalized expression it was observed that local leaf expression was higher for all three tested soil treatments compared to the unexposed or systemic leaves or both after *P. rapae* and *M. brassicae* feeding (**figure 3.12** and **figure 3.13**). These results might indicate that six hours of larval exposure time is sufficient for insect induced resistance related to *BoLOX* expression. An effect of priming was not observed for *BoLOX*, in contrast to earlier findings (Zheng et al., unpublished results).
BoLOX in CHA0 systemic leaves was significantly higher expressed for *M.brassicae* compared to *P.rapae* feeding (P=0.036) (**figure 3.14**). This small significant result might be caused by experimental defects as this observation is not detected in the other treatments.

Similar results as *BoLOX* normalized expression were observed for JA transcription factor *BoMYC* (Boter et al. 2004) only the last mentioned displayed a ten-fold higher overall normalized exspression. *BoMYC* local leaf expression was higher compared to either control or systemic leaf or both for all different soil treatments for after *P.rapae* and *M.brassicae* exposure (**figure 3.15** and **figure 3.16**). This is in consensus with the theory that herbivorous insects induce plant defense upon feeding (Van Poecke and Dicke, 2002). Expression in systemic leaves was higher for *Pf* CHA0 and CHA1144 compared to undamaged leaf after *M.brassicae* feeding and for CHA1144 after *P. rapae* feeding. This might indicate that six hours of insect exposure is suitable to induce systemic resistance of *BoMYC*.

However, systemic resistance is still significantly lower in the mutant strain compared to the control strain after *P.rapae* exposure. In contrast to previous findings on priming of *BoMYC* (Zheng et al. unpublished results) no priming was observed here.

For *BoMYR* we only observe an up-regulation in gene expression after *P. rapae* feeding in the local leaves of plants induced by CHA0 (**figure 3.19**). No effect of priming was observed (**figure 3.18** and **figure 3.19**). This is consistent with earlier results after infestation of *B.oleracea* with WCS417r (Zheng, unpublished results). Our results might indicate that expression levels in *BoMYR* are not changed to great extent after *P.rapae* and *M.brassicae* feeding.

Here, we tested a few of many plant resistance genes. To obtain a better inside in plant gene modification after PGPR wild and mutant *Pf* infestation upon caterpillar attack, it might be plausible to perform micro-array studies, where an overview of the entire *B.oleracea* genetic profile can be displayed. This might elucidate, which pathways are involved in PGPR ISR in relation to underlying plant-insect interactions. To improve and extent the information obtained from qRT-PCR results, I suggest that future experiments are conducted at different time points of 6h, 24h, 48h and 72h of total insect exposure. Experimental tests for different influencing biological factors must be conducted to improve prospective methodology for qRT-PCR, since little information is currently available on the level of insect quantitative gene expression.

In conclusion, we did not observe priming for either specific tested *B.oleracea* gene, which might be caused by either the absence of priming or a deficient experimental approach, which needs improvement. The last mentioned explanation is most likely, since it was observed by Iavicoli et al., (2003) that disease suppression by means of ISR in the presence of root-colonizing *Pf* CHA0 in *A. thaliana* oomycete *Peronospora parasitica* infested plants, was absent in mutants insensitive to ethylene in the roots, insensitive to jasmonic acid and methyl jasmonic acid and mutants that were not expressing the NPR1 protein. According to these observations and previous observations (Zheng et al, unpublished results) where it was observed that *BoPRI, BoMYC* and *BoLOX* were primed by *P.fluorescens* strain WCS417r in *B.oleracea*, it is expected that the JA pathway and presumably also the SAR pathway are involved in *Pf* induced ISR. If priming occurs in *B.oleracea* infested with either *Pf* CHA0 or CHA1144 or both after caterpillar damage, it would explain our observations from the larval body weight gain experiment and the Y-tube olfactometer experiment, where we observed that the presence of Pseudomonads strains CHA0 and CHA1144 has an influence on direct and indirect plant defence. However, the physiological underlying mechanism for this phenomenon still needs to be elucidated.

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