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Department of Plant biology
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Abstract

Beneficial bacteria colonizing plant roots are capable of mediating improved plant growth and enhanced defense responses against biotic and abiotic stresses. Such colonization has been suggested to accumulate brassinosteroid (BR) levels but the mechanisms behind this is not yet completely understood. This study confirms that *Bacillus amyloliquefaciens* UCMB5113 is capable of promoting growth in *Arabidopsis thaliana* wild type Col-0 and BR deficient *det2-1* mutants. These results suggest that *Bacillus* potentially promotes growth by elevating the endogenous BR levels which further led to the proposal that *Bacillus* releases a protein interacting with the BR receptor BRI1 triggering BR dependent signaling. This interaction remains to be established since no such protein was identified in the performed immunoprecipitation. Gene expression analysis revealed that *Bacillus* was only able to mediate primed defense responses as induced systemic resistance (ISR) in Col-0 but not in *det2-1* plants upon challenge with the fungal pathogen *Alternaria brassicicola*. Since certain BR levels are essential in defense responses of plants, it was suggested that *Bacillus* was not capable of elevating BR to levels high enough to provide protection against *A. brassicicola*. GUS assays showed systematic expression of *BAK1* in leaves of *Bacillus* treated plants. Further, the expression was observed to relocate to the inoculation site upon pathogen challenge. These findings suggest a role for BAK1 both in root colonization of *Bacillus* and defense. *BRI1* was also shown to be induced by *Bacillus* and interestingly, also by a suspension of *Bacillus* cell walls. This reveals that non-viable bacteria can stimulate *BRI1* expression, but it remains to be determined whether non-viable bacteria can mediate primed growth and defense as well. Gene expression analysis identified a probable role for *ML* genes (1,3,5,8) during colonization of *Bacillus*. However, preliminary studies performed on *ml3-1* and *ml7-1* mutants did not indicate that they have any major role in ISR.

Keywords: Arabidopsis thaliana, *Bacillus amyloliquefaciens* UCMB5113, Biocontrol, Brassinosteroids, Induced systemic resistance, Priming.

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1 Introduction

1.1 Beneficial bacteria mediated priming of plant defense and enhanced growth

Soil contains complex microbiota composed of a wide range of microorganisms some of which are capable of interacting with plants in the rhizosphere. The established interactions can affect plants in different ways depending on the lifestyle of the colonizing microbe. Some bacterial strains present in soil are regarded as beneficial since they are able to promote plant growth and enhance defense responses against biotic and abiotic stresses (Bejai *et al*., 2009; Danielsson, 2008). This plant protecting process caused by prior treatment with beneficial bacteria is referred to as priming and results in enhanced defense responses upon stress challenge (Conrath *et al*., 2006; Van der Ent *et al*., 2009). A previous study demonstrated that the rhizobacterium *Bacillus amyloliquefaciens* strain UCMB5113 was able to protect oilseed rape (*Brassica napus*) against several fungal pathogens (Danielsson *et al*., 2006). It has also been shown that *B. amyloliquefaciens* is capable of protecting plants against abiotic stresses. A recent study reported that *B. amyloliquefaciens* UCMB5113 was able to improve heat stress tolerance in wheat (*Triticum aestivum*) (El-Daim *et al*., 2014). In this study, *B. amyloliquefaciens* UCMB5113 was used to treat *Arabidopsis thaliana* plants in order to elucidate mechanisms behind the enhanced defense response and promoted growth.

1.2 Defense response upon stress

The immobile state of plants has resulted in the development of various systemic defense responses where plants depend on chemical signaling that emerges systemically from the site of infection when the innate immunity system is activated. Systemic responses make plants capable of defending themselves against abiotic and biotic stresses in their surrounding environment. The plant immune system is

essentially divided into two parts. The primary response is initiated after molecular recognition of pathogens which induces a rapid local hypersensitive response (HR) that commonly leads to cell death at the infection site. The secondary response is mediated by resistance-gene encoded recognition of pathogenic virulence factors which leads to induction of resistance against intrusive pathogens (Jones and Dangl, 2006; Sadava *et al*., 2011). Signaling pathways associated with defense processes in plants are mediated by phytohormones like salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Colonization of biotrophic pathogens are known to activate the SA-signaling pathway which mediates the systemic acquired resistance (SAR). SA-signaling is required for activating genes that encode pathogenesis-related (PR) proteins with antimicrobial properties effective against biotrophic pathogens and insects. Whereas JA and ET mediate the induced systemic resistance (ISR) elicited by root colonizing rhizobacteria. JA-signaling is known to induce defense responses against necrotrophic pathogens and insects (Ton, *et al*., 2002; Van der Ent *et al*., 2009; Van Loon, *et al*., 1998). Priming mediated by *B. amyloliquefaciens* UCMB5113 has previously been shown to elicit ISR in oilseed rape upon pathogen challenge (Bejai *et al*., 2009).

As mentioned, the molecular recognition of intrusive pathogens is known to initiate defense responses in plants. The plant may recognize conserved molecular features on the entering microbes by pattern recognition receptors (PRRs) that are localized in the cell membrane (Jones and Dang, 2006). These features are referred to as microbe-associated molecular patterns (MAMPs) which upon recognition activates a signaling cascade mediated by JA and ET which further induces ISR (Millet *et al*., 2010). Many PRRs are conserved evolutionary and belong to the Toll-like receptor (TLR) family. A well-known PRR is the flagellin receptor (FLS2) recognizing bacterial flagellin proteins building up flagella. Often a synthetic peptide (flg22) corresponding to a small portion of the protein is used as ligand to study receptor activation. This receptor has been proposed to interact and form complex with BAK1 (BRI1-associated receptor kinase 1) after flg22 binding (Chinchilla *et al*., 2007). BAK1 is mainly associated with BRI1 (brassinosteroid insensitive 1) which is a brassinosteroid (BR) receptor involved in plant development (Sadava *et al*., 2011). Even though the output of BRI1 and FLS2 is distinct, BAK1 is proposed to co-regulate them both. BAK1 has therefore also been suggested to be involved in plant immunity processes (Chinchilla *et al*., 2007).

In addition, ML (MD2-related lipid recognition) proteins are known to promote innate immunity in mammals, however, the knowledge regarding their role in plants is still limited (Inohara and Nuñez, 2002). Recent studies have demonstrated that the *ML* gene family is participating in defense responses in *A. thaliana* against

insects and pathogens (Fridborg *et al*., 2013; Hakenjos *et al*., 2013). Another study has identified up-regulation of *ML5* and *ML9* in *A. thaliana* roots after treatment with the well-studied biocontrol agent *Pseudomonas fluorescens* (Van de Mortel *et al*., 2012). Selected *ML* gene family members were therefore studied in this project to understand their role in plant defense responses in plants treated with *B. amyloliquefaciens* UCMB5113.

1.3 Brassinosteroids

BRs belong to the steroidal class of phytohormones which have a fundamental role in development processes associated with growth regulation. BRs are known to participate in events like cell division and expansion, stem elongation, vegetative growth, root inhibition and leaf bending (Sasse, 2003; Sadava *et al*., 2011). It is also known that BRs are involved in plant innate immunity via the involvement of BAK1 in MAMP recognition (Chinchilla *et al*., 2007). Another study has shown that BRs are involved in stress responses against abiotic and biotic stress factors such as pathogens and cold although the underlying mechanisms are not completely understood (Nakashita *et al*., 2003). In addition, a transcript profiling analysis reported that BR associated genes are up-regulated in plants treated with *B. amyloliquefaciens* UCMB5113 (Bejai *et al*., 2009).

The BR signaling is initiated when a BR molecule binds to the BRI1 receptor which is situated in the plasma membrane of plant cells. The main BR is referred to as brassinolide which is synthesized from the precursor campesterol. The synthesis can occur both via the late C-6 oxidation pathway and the early C-6 oxidation pathway. BRI1 binding occurs at high levels of BRs which results in autophosphorylation of the C-terminal domain of BRI1 leading to dissociation of the inhibitor protein BKI1 (BRI1 Kinase Inhibitor 1) (Divi and Krishna, 2009; Sadava *et al*., 2011). Thus, BRI1 can associate with its co-receptor BAK1 and through transphosphorylation cause an increased signaling output (Wang *et al*., 2008). The transcription factors, BZR1 and BES1, will either be activated or suppressed as a result of the present BR levels. In absence of BR, BIN2 (brassinosteroid-insensitive 2) negatively regulates BZR1 and BES1 through phosphorylation. In contrast, activation of BSU1 (bri1 suppressor 1) occurs when BR levels are high leading to positive regulation through dephosphorylation of BZR1 and BES1. The active state of BZR1 and BESI further leads to downstream regulation of associated BR genes (Divi and Krishna, 2009; Wang and He, 2004). The main hypothesis of this investigation is that *B. amyloliquefaciens* UCMB5113 treatment of *A. thaliana* roots lead to accumulated BR levels in the plant which will promote growth and trigger defense responses upon biotic stress.

2 Aims

The main aims of this project were

1) To elucidate the role of BRs in growth promotion and accumulated defense responses mediated by *B. amyloliquefaciens* UCMB5113. The project also attempted to investigate the role of BR receptors BAK1 and BRI1 during root colonization of UCMB5113 and evaluate their role in primed defense responses.

2) To identify a potential MAMP associated with *B. amyloliquefaciens* UCMB5113 triggering BR signaling.

3) To explore the role of *ML* gene family members in *Bacillus* mediated priming of plant defense.

3 Materials and methods

3.1 Plant material

Wild type Columbia (Col-0) and transgenic lines of *A. thaliana* were used in this study to elucidate the mechanisms of *B. amyloliquefaciens* UCMB5113 mediated priming of plant growth and defense response. The transgenic lines *bri1-1* and *bak1-4* used are defective in BR signal transduction due to inoperative receptors (Chinchilla *et al*., 2007) while *det2-1* plants are defective in the BR biosynthetic pathway (Noguchi *et al*. 1999). Mutant lines *ml3-1* and *ml7-1* (Hakenjos *et al*., 2013; Fridborg *et al*., 2013) were used in *ML* gene family studies. β-Glucoronidase (GUS) transgenic lines *ML3p:GUS* (Hakenjos *et al*., 2013) and *BRI1:GUS* (Jeong *et al*., 2010) were used for histochemical gene expression studies and a green fluorescent protein (GFP) transgenic line *BAK1:GFP* (Jeong *et al*., 2010) was used in immunoprecipitation experiments.

3.2 Bacteria

B. amyloliquefaciens UCMB5113 is a soil-isolated rhizobacterium which produces endospores that are used in suspension when plants are treated to prime growth and defense responses. *B. amyloliquefaciens* UCMB5113 colonizes the root efficiently by constructing surface structures enabling it to adhere to root and seed surfaces (Reva *et al*., 2004). In this study, *Bacillus* treatment was conducted by soaking roots of *A. thaliana* in *B. amyloliquefaciens* UCMB5113 spore suspension (1×10^7 CFU mL^{-1}).

3.3 Pathogen

The necrotrophic fungal pathogen *Alternaria brassicicola* MUCL20297 was used in pathogen assays in order to evaluate the ability of *B. amyloliquefaciens*

UCMB5113 to mediate priming of defense responses upon challenge. The fungal pathogen was grown on potato dextrose agar plates for 2 weeks at 22°C. Fresh fungal spore suspension (5 x 10^5 ml^{-1}) was applied on two leaves per plant as described previously (Thomma *et al*., 1998).

3.4 To elucidate the role of BRs in plant growth and stress response upon *B. amyloliquefaciens* UCMB5113 treatment

3.4.1 Effect of *Bacillus* treatment on BR deficient plants

A. thaliana seeds (Col-0 and *det2-1*) were surface sterilized in 70 % ethanol for 10 seconds followed by incubation in 10 % chlorine and a little Tween 20 for 5 minutes and later washed three times with sterile water. The sterilized seeds were germinated on MS agar medium in controlled environment at 22°C day and 20°C night, 18/6 h (day/night), fluorescent light intensity of 110 µmol $m^{-2}s^{-2}$, 70 % RH. Six days old plants were treated by soaking the roots in *B. amyloliquefaciens* UCMB5113 spore suspension (1 x 10^7 CFU mL^{-1}). Control plants were treated with distilled water. One set of *Bacillus* treated and control plants were arranged in triplicates (4 plants per plate) on MS agar medium, the plants were grown under equal growth conditions to those described. The root architecture and leaf phenotype were analyzed eight and 14 days later.

The rest of the *Bacillus* treated and control plants were grown in standard soil (S-jord, Hasselfors garden) on separate trays containing 20 plants/mutant which were then grown in controlled conditions at 22°C day and 20°C night, 18/6 h (day/night), fluorescent light intensity of 200 µmol $m^{-2}s^{-2}$, 70 % RH. A set of plants were harvested after two weeks and sent for BR analysis to the Laboratory of Plant Growth Regulator, Olomouc, Czech Republic. The other set were followed up to flowering (three weeks after *Bacillus* treatment) for phenotypic analysis.

3.4.2 Effect of *Bacillus* treatment upon pathogen challenge in BR deficient plants

One-week-old *A. thaliana* plants (Col-0 and *det2-1*) grown on MS agar medium were root treated with *Bacillus* spore suspension (1 x 10^7 CFU mL^{-1}) and control plants treated with water. These plants were inoculated with *A. brassicicola* two weeks later. Fresh fungal spore suspension (5 x 10^5 ml^{-1}) was used to inoculate four leaves per plant where each leaf was inoculated with 5 µl spore solution. Disease symptoms were recorded after five days. The inoculated leaves of *Bacillus* treated and control plants were harvested and snap frozen in liquid nitrogen and stored at -20°C.

3.4.3 Quantitative real-time PCR (qPCR) analysis of pathogen

The frozen leaf samples (*det2-1*, Col-0) were pulverized by using a tissue lyser MM200 (Retsch). DNA extraction was performed according to the manufactures manual using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific) with the exception that 30 µl elution buffer was used instead of 100 µl when eluting the DNA. The quantity and quality of the DNA was determined by the A260/A280 ratio using a Nanodrop ND-1000 (Fischer Science).

The qPCR quantification was performed using the Maxima SYBR GREEN qPCR Master Mix (2x) kit (Fermentas), run in a MyIQ cycler (BioRad) using the software BioRD iQ5. Each qPCR reaction (20 µl) contained 10 µl PCR SYBR Green Master Mix, 1.2 µl (5 µM) forward primer and reverse primer (Table 1) and 2.6 µl sterile water. 5 µl DNA (5 ng μl^{-1}) was used as template in each reaction. Negative controls were supplemented with the equal amount of sterile water. Three technical replicates were used during the analysis and the reactions were performed in a 96-well optical reaction plate (Applied Biosystems). The cycling condition of the reaction was as follows; 7 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 60°C, 1 min at 95°C, 1 min at 60°C, and 71 cycles of 10 s at 95°C and 20 s at 60°C.

Primer3 software was used (Koressaar and Remm, 2007) to design primers associated with the strain specific cutinase gene (*AbrCUT*) present in *A. brassicicola* in order to quantify *A. brassicicola* DNA relative to *A. thaliana* DNA (*ubiquitin5*) in inoculated leaves. Ubiquitin5 (*UBQ5*) was used as a reference gene and its threshold cycle (CT) values were used to normalize the data.

Student's t-test was conducted in Microsoft Excel software using the qPCR data in order to analyze statistically significant differences between the *Bacillus* treated and control leaves upon pathogen challenge.

Table 1. Primers used in qPCR quantification of pathogen.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
UBQ5 (At3g62270)	CGATGGATCTGGAAAGGTTC	AGCTCCACAGGTTGCGTTAG
AbrCUT	ATCACTGCCGGTGGTTACTC	CGACACCCTTGATTTGGTCT

3.5 To elucidate the role of BR receptors BAK1 and BRI1 during *B. amyloliquefaciens* UCMB5113 colonization of *A. thaliana* roots and enhanced plant defense

Two weeks old *A. thaliana* plants (Col-0, *bak1-4* and *bri1-1*) grown on MS agar medium under equal conditions to those described earlier were treated by soaking the roots into *B. amyloliquefaciens* UCMB5113 suspension (1 x 10^7 CFU mL^{-1}). Control plants were treated with distilled water. *Bacillus* treated and control plants were arranged in triplicates (4 plants per plate) on MS agar plates. The plants were grown under equal growth conditions to those described. The plants were inoculated with *A. brassicicola* spore solution (5 x 10^5 ml^{-1}) one week after the event of root treatment. Disease development was recorded after one week post-inoculation.

3.6 To identify a potential MAMPs of *B. amyloliquefaciens* triggering BR signaling

3.6.1 A time-course study on *BAK1:GUS* plants after treating with *Bacillus* and challenge inoculation with *A. brassicicola*

One week old *A. thaliana* plants (*BAK1:GUS*) grown on MS agar medium under equal conditions to those described were treated by *B. amyloliquefaciens* UCMB5113 suspension (1 x 10^7 CFU mL^{-1}). Control plants were treated with distilled water. A set of plants were collected at different time points (1-5 days) post-treatment and stained for GUS expression for 1 h at 37°C and later destained using 70 % ethanol. Spatial gene expression was analyzed using confocal microscopy.

The other set of *Bacillus* treated and control plants were subsequently grown for 12 days, four leaves per plant were then inoculated with *A. brassicicola* spore solution (5 x 10^5 ml^{-1}). The inoculated and non-inoculated leaves were collected two days post-inoculation and stained for GUS expression as described above.

3.6.2 A time-course study on *BRI1:GUS* plants after pre-treatment with *Bacillus* cell wall suspension

Preparation of bacterial cell walls was conducted by sonication according to Van Wees *et al*. (1997). Roots of one week old *A. thaliana* plants (*BRI1:GUS*) grown on MS agar medium were treated with 20 µl of intact cell suspension as well as sonicated cell wall suspension of *B. amyloliquefaciens* UCMB5113 (1 x 10^7 CFU mL^{-1}). Control plants were treated with distilled water. Two plants per treatment were collected during four time points (24 h, 48 h, 3 days and 8 days) post-treatment. The plants were stained for GUS expression for 1 h at 37°C and later

destained using 70 % ethanol. Spatial gene expression was analyzed using confocal microscopy.

3.6.3 Immunoprecipitation of *BRI1:GFP* after *Bacillus* treatment

Roots of one week old *A. thaliana* plants (*BRI1:GFP*) grown on MS agar were treated with *B. amyloliquefaciens* UCMB5113 suspension (1 x 10^7 CFU mL^{-1}). Control plants were treated with distilled water. The plants were arranged in duplicates (10 plants per treatment) on MS agar and grown under the same growth conditions to those described. The roots were collected two weeks post-treatment and homogenized separately in extraction buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 0.2% 2-mercaptoethanol, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail. The homogenized plant materials were centrifuged for 10 min at 10,000 g the supernatants were incubated with 5 µl anti-GFP antibody (Invitrogen) at 2°C overnight with gentle shaking. Subsequently 50 µl of Protein A Sepharose (Invitrogen) was added to the protein complexes and incubated for at 2°C for 3 h with gentle shaking. Immunoprecipitated complexes were collected by centrifugation at 1,500 g for 2 min and repeated (3x) washing in 1 ml PBST (0.05 % Tween 20). The precipitated protein complexes were separated by SDS-PAGE and visualized via western blotting using GFP-specific antibodies (Invitrogen).

3.7 To investigate the role of *ML* gene family in *Bacillus* mediated priming of plant defense

3.7.1 qPCR analysis of *ML* genes

Expression of *ML* genes in *A. thaliana* was screened for using qPCR analysis as described previously with *ML* gene-specific primers (Table 2). cDNA was generated from *Bacillus* treated and control plants of *A. thaliana* Col-0. cDNA was obtained from a previous study in the lab. Student's t-test was conducted in Microsoft Excel software using the qPCR data in order to analyze statistically significant differences in gene expression between the *Bacillus* treated and control leaves.

Table 2. Primers used in qPCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ML1 (At3g11780)	TTATTTTCTTGGTTTCAACGATT	TTCGTTATTATCGCAGTAGTGAACA
ML2 (At3g44100)	TGGTTCTACTGGCGAAGACA	TCCCAACGTATAAAACCCTGA
ML3 (At5g23820)	TTCCGACGTTCTTTATGTTGG	ACTTTGAAGTCGACACACATTTTTA
ML4 (At5g06480)	GGGAAGCTGGTGATGAAGT	TCTCAGTGCAGAGGTCATGAGT
ML5 (At5g23849)	GAATCAAGTATTCGGTATCTTTGC	CGACACACATTTTTATTATTGGATTC
ML7 (At2g26370)	GTTTGCAGTCACTCCCTCATAA	TTTAGCAGTGGCACTTCAGC
ML8 (At1g45015)	CGAAATCTCACTACCAGCCTCT	CGTATCCAATACCGTTGCAG
ML9 (At2g16005)	CCCGATTGTGCTTACTCTCC	GAGGCTTATCGGTGATGCTT

3.7.2 Efficiency of *Bacillus* treatment on *ml3-1* and *ml7-1* mutants upon *A. brassicicola* inoculation

A. thaliana plants (Col-0 wild type, and T-DNA knockout lines *ml3-1* and *ml7-1*) were grown on standard soil (S-jord, Hasselfors garden) for one week before adding 1 mL *B. amyloliquefaciens* UCMB5113 suspension (1 x 10^7 CFU mL^{-1}) to the soil near the rosettes. Control plants were treated with sterile water. *Bacillus* treated and control plants were grown in separate trays in a growth chamber using conditions described above. Leaves were inoculated with *A. brassicicola* spore solution (5 x 10^5 ml^{-1}) as described one week after *Bacillus* treatment. Disease development was recorded after five days post-inoculation.

3.7.3 A time-course study on *ML3p:GUS* plants after *Bacillus* treatment and challenge inoculation with *A. brassicicola*

Ten days old *A. thaliana* plants (*ML3p:GUS*) grown on MS agar were treated with *B. amyloliquefaciens* UCMB5113 suspension (1 x 10^7 CFU mL^{-1}). Control plants were treated with distilled water. Leaves of the plants were inoculated with *A. brassicicola* spore solution (5 x 10^5 ml^{-1}) two days after *Bacillus* treatment. Roots, inoculated and non-inoculated leaves were collected two days post-inoculation and stained for GUS expression for 1 h at 37°C and later de-stained using 70 % ethanol. Spatial gene expression was analyzed using confocal microscopy.

An additional GUS assay was performed where cell wall suspension of *B. amyloliquefaciens* UCMB5113 was included in the root treatment. Roots of one week old *A. thaliana* plants (*ML3p:GUS*) grown on MS agar were treated with 20 µl per root of intact and cell wall *B. amyloliquefaciens* UCMB5113 suspension (1 x 10^7 CFU mL^{-1}). Control plants were treated with distilled water. Two plants per treatment were collected during three time points (24 h, 4 days and 8 days) post-treatment. The plants were stained for GUS expression before analysis using confocal microscopy as described.

4 Results

4.1 The role of BRs in plant growth and stress response upon *B. amyloliquefaciens* UCMB5113 treatment

4.1.1 Effect of *Bacillus* treatment on BR deficient plants

A phenotypic study was done in order to study the role of BRs in *B. amyloliquefaciens* UCMB5113 mediated priming of plant growth and defense responses. The phenotypic study was performed using wild type Col-0 and BR deficient mutant *det2-1* plants. Roots were treated with *B. amyloliquefaciens* UCMB5113 and controls were treated with distilled water. The root and leaf phenotype were recorded eight and 12 days post-treatment. The flowering phenotype was recorded three weeks post-treatment.

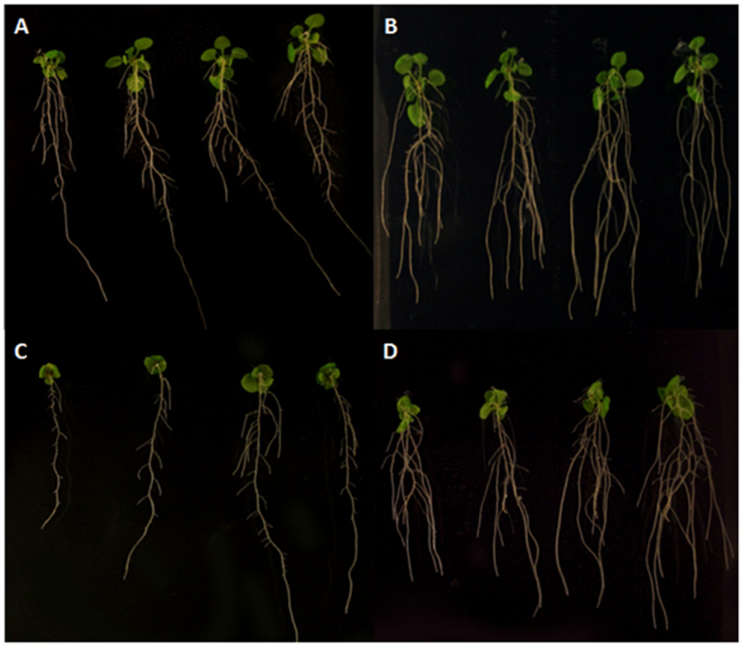

Figure 1. Eight days after *Bacillus* treatment. (A) Col-0 control, (B) Col-0 treated, (C) *det2-1* control and (D) *det2-1* treated.

The results from the phenotypic study of *A. thaliana* plants (Col-0 and *det2-1*) eight days post-treatment with UCMB5113 showed a clear distinction in the root morphology of the non-treated and the treated plants. Col-0 control plants (Figure 1A) had less lateral roots in comparison with Col-0 treated plants (Figure 1B). The leaves of treated plants were generally a bit larger than leaves of control plants. This phenotypic distinction was also observed between *det2-1* control plants (Figure 1C) and *det2-1* treated plants (Figure 1D).

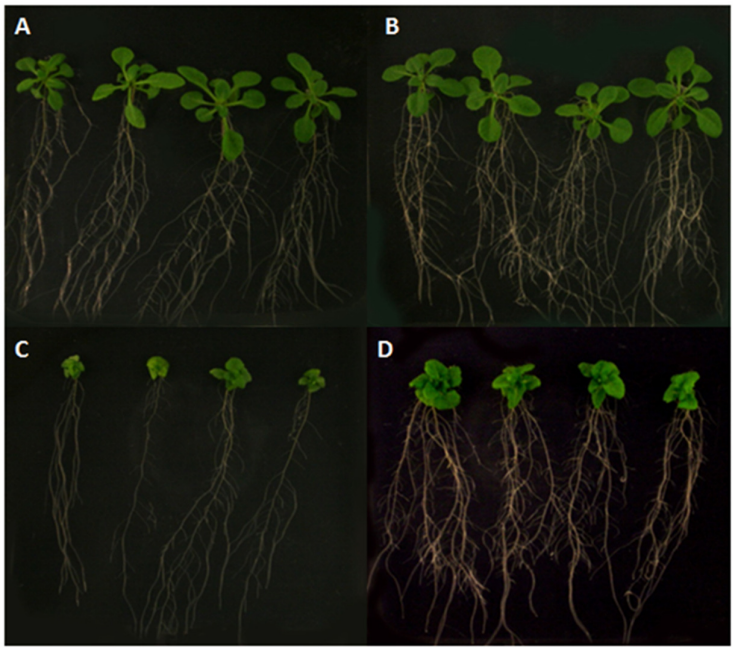

Figure 2. *A. thaliana* plants 12 days after *Bacillus* treatment. (A) Col-0 control, (B) Col-0 treated, (C) *det2-1* control and (D) *det2-1* treated.

The results from the phenotypic study of *A. thaliana* plants (Col-0 and *det2-1*) 12 days post-*Bacillus* treatment showed a less clear distinction concerning the lateral roots and leaves of control Col-0 plants (Figure 2A) and *Bacillus* treated Col-0 plants (Figure 2B). The treated Col-0 plants had more lateral roots but the size of the leaves was equal to leaves of control Col-0 plants. A phenotypic distinction was more clear between *det2-1* control plants (Figure 2C) and *det2-1* treated plants (Figure 2D). The treated *det2-1* plants had more lateral roots and larger leaves in comparison with the control plants of *det2-1*.

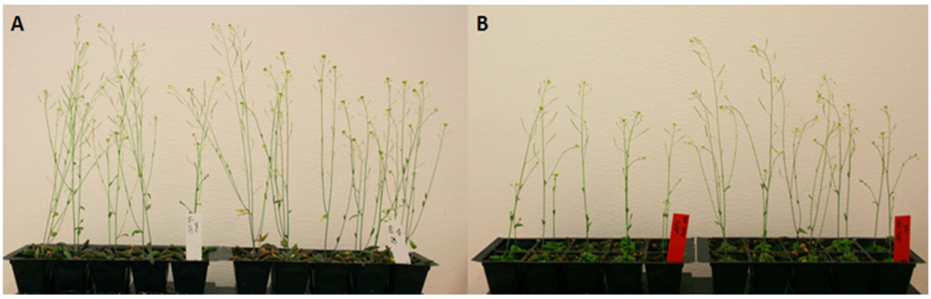

Figure 3. *Bacillus* treated *A. thaliana* plants, three weeks post-treatment. (A) From left to right: Col-0 control and Col-0 treated. (B) From left to right: *det2-1* control and *det2-1* treated.

There was no notable difference between the phenotype of Col-0 control plants (Figure 3A, left) and Col-0 UCMB5113-treated plants (Figure 3A, right). However, the plants of the treated *det2-1* mutants (Figure 3B, right) were higher than the control *det2-1* plants (Figure 3B, left).

No results were obtained from the BR analysis due to technical problems with instruments. Therefore, no results of the BR levels present in *Bacillus* treated plants respective control plants are presented.

4.1.2 Effect of *Bacillus* treatment upon pathogen challenge in BR deficient plants

A pathogen assay was done in order to examine the role of BRs in *B. amyloliquefaciens* UCMB5113 mediated defense responses. The necrotrophic pathogen *A. brassicicola* was used to challenge control and *Bacillus* treated plants (Col-0 and *det2-1*) two weeks post-treatment. Disease symptoms were recorded after five days.

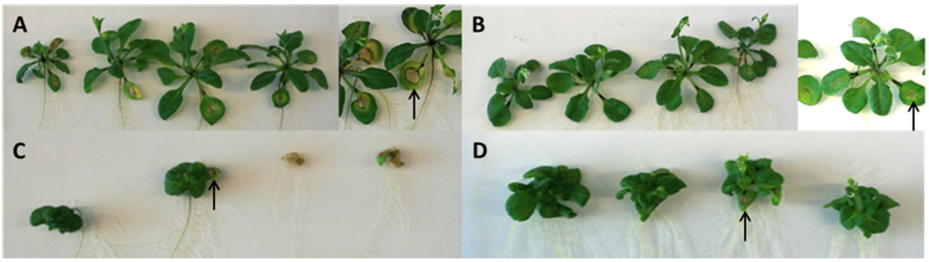

Figure 4. *A. thaliana* plants five days after inoculation with *A. brassicicola*. (A) Col-0 control, (B) Col-0 *Bacillus* treated, (C) *det2-1* control and (D) *det2-1 Bacillus* treated.

The results from the pathogen assay showed that necrotic lesions present in Col-0 control plants (Figure 4A) were larger in comparison with Col-0 treated plants (Figure 4B). It appears like *B. amyloliquefaciens* UCMB5113 is able to mediate primed defense responses against *A. brassicicola* in Col-0 plants to some extent. Two control *det2-1* plants were not viable after the pathogen assay and the inoculated leaves had visible necrotic lesions (Figure 4C). In contrast, all treated *det2-1* plants were viable after the inoculation. The treated *det2-1* mutants had necrotic lesions equally to those observed in *det2-1* mutants under control conditions (Figure 4D).

4.1.3 qPCR quantification of pathogen

The assessment of *B. amyloliquefaciens* UCMB5113 ability to mediate priming of defense responses was further performed by quantifying *A. brassicicola* DNA (*cutinase*) relative to the *A. thaliana* DNA (*ubiquitin5*). The qPCR quantification

was done with leaves of *Bacillus* treated and non-treated *A. thaliana* (Col-0 and *det2-1*) plants. The leaves were inoculated with *A. brassicicola* two weeks post-treatment and collected five days after the event of inoculation. Control plants were treated with distilled water.

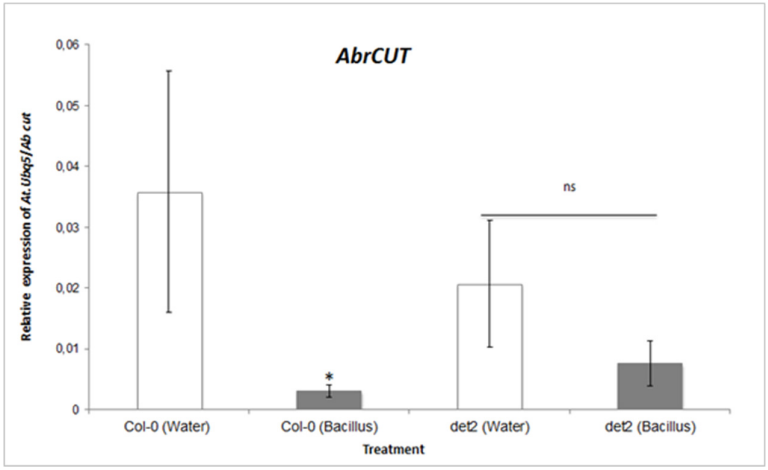

Figure 5. qPCR quantification of *A. brassicicola* DNA (*cutinase*) relative to *A. thaliana* DNA (*ubiquitin5*). Significance level of data: * $p < 0.05$ and ns (non-significant) $p > 0.05$.

Col-0 leaves were found to accumulate 8-fold higher fungal DNA levels in the control plants compared to treated plants (Figure 5). There is a significant difference between these two conditions indicating that *B. amyloliquefaciens* UCMB5113 potentially mediate primed defense responses when plants are challenged with *A. brassicicola*. However, the data of Col-0 control contains a large standard deviation which gives the significance some uncertainty. There is no significant difference between the relative expression of *AbrCUT* in *det2-1* under control conditions and *det2-1* under primed conditions when challenged with *A. brassicicola*.

4.2 The role of BR receptors BAK1 and BRI1 during *B. amyloliquefaciens* UCMB5113 colonization of *A. thaliana* roots and enhanced plant defense

In order to understand the role of BR receptors BAK1 and BRI1 in *B. amyloliquefaciens* UCMB5113 mediated priming of defense response, the mutant lines *bak1-4* and *bri1-1* treated or not with *Bacillus* were challenged with *A. brassicicola*. One week post-treatment, leaves of mutant lines and Col-0 were inoculated with *A. brassicicola*. The disease development was visually recorded one week after inoculation.

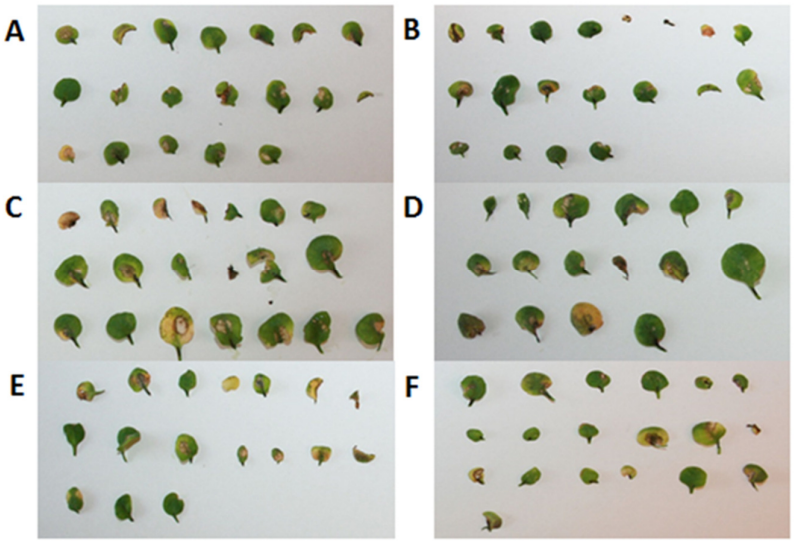

Figure 6. Disease development one week after inoculation with *A. brassicicola*. (A) Col-0 control, (B) Col-0 *Bacillus* treated, (C) *bak1-4* control, (D) *bak1-4 Bacillus* treated, (E) *bril-1* control and (F) *bril-1 Bacillus* treated.

No obvious visual difference in disease development was observed one week post-inoculation (Figure 6). Leaves of Col-0, *bak1-4* and bril-1 showed similar quantities of necrotic lesions among the *Bacillus* treated and water treated controls.

4.3 Identification of potential MAMPs of *B. amyloliquefaciens* UCMB5113 triggering BR signaling

4.3.1 A time-course study on *BAK1:GUS* plants after treatment with *Bacillus* and challenge inoculation with *A. brassicicola*

A GUS reporter gene assay was carried out in order to study *BAK1* expression in plants treated with *B. amyloliquefaciens* UCMB5113. Plants were collected and stained at different time points (1-5 days) post-treatment with *Bacillus*. Control plants were treated with distilled water. Further, plants were inoculated with *A. brassicicola* 12 days post-treatments in order to assess *BAK1* expression in challenged plants under *Bacillus* treatment and control conditions. The inoculated and non-inoculated leaves were collected and stained two days post-inoculation.

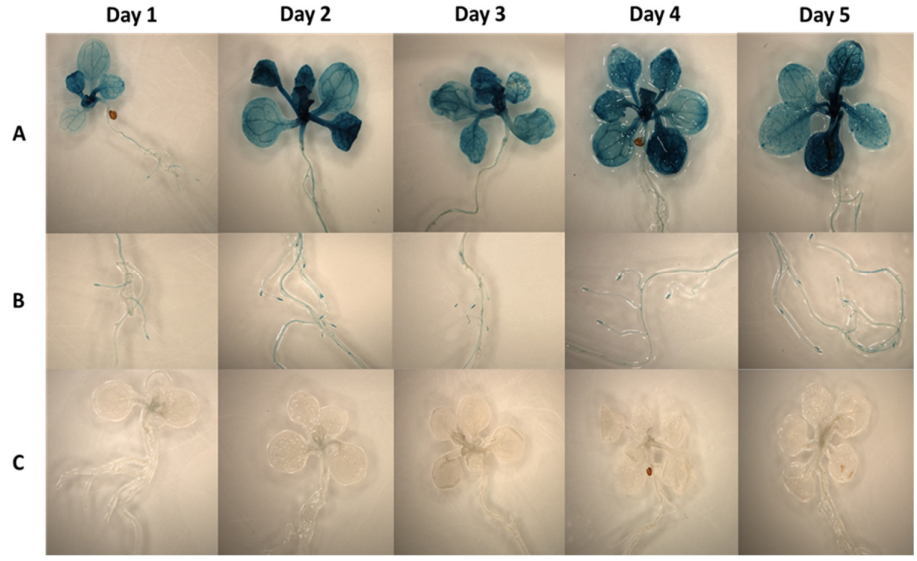

Figure 7. A time-course expression analysis of *BAK1:GUS* in roots and leaves after *Bacillus* or water treatments. (A) *Bacillus* treated, (B) Roots of *Bacillus* treated plants, and (C) Control plants.

BAK1 expression was shown to be expressed at a high level over the whole rosette already at day 1 after *Bacillus* treatment (Figure 7A). The expression increased slightly at day 2 and thereafter, it remained at a similar level to day 5. The expression level of *BAK1* was shown to be highest in the cotyledons during the whole time-course among the leaves. The expression was also shown to remain high in the shoot meristem. The expression of *BAK1* was systemic over the entire plant and not limited to the root area where the *Bacillus* treatment was done (Figure 7A,B). Interestingly, the root tips of the secondary roots had the highest levels of *BAK1* expression whereas the primary roots had a slightly lower expression level. Only a faint staining was observed in the control plants during the time-course (Figure 7C).

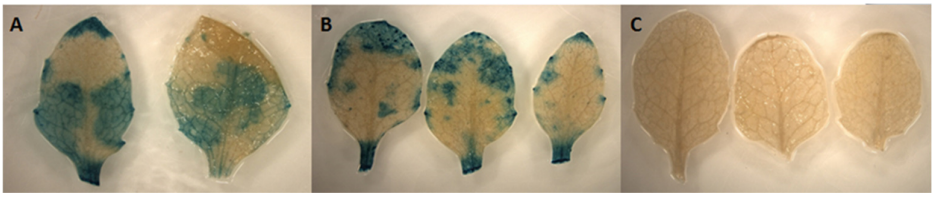

Figure 8. Expression analysis of *BAK1:GUS* two days after *A. brassicicola* inoculation on *Bacillus* treated *BAK1:GUS* plants. (A) Inoculated leaves, (B) Non-inoculated leaves and (C) Control (inoculated and non-inoculated).

The results from the pathogen assay showed that *BAK1* expression was located mainly to the center of the leaves (Figure 8A). The centric pattern corresponds to the site where the pathogen inoculation was performed. *BAK1* was also shown to be slightly expressed at the leaf margin. In the non-inoculated leaves, *BAK1* was mainly expressed at the margin and apex with high levels at hydathodes (Figure 8B). Weak *BAK1* expression was observed in the controls during pathogen assay (Figure 8C).

4.3.2 A time-course study on *BRI1:GUS* plants after pre-treatment with *Bacillus* cell wall suspension

An additional GUS reporter gene assay was carried out in order to study how *BRI1* is expressed in plants treated with *B. amyloliquefaciens* UCMB5113. In this experiment, a cell wall suspension of *B. amyloliquefaciens* UCMB5113 was used in order to compare potential expression differences of *BRI1* in plants treated with intact cells. Plants were collected and stained at different time points (1-3 days) post-treatment. Control plants were treated with distilled water.

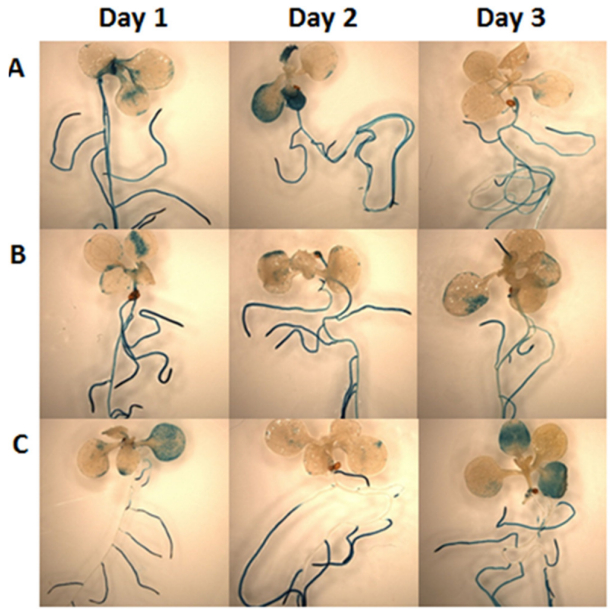

Figure 9. A time-course study on *BRI1:GUS* plants treated with intact cells or a cell wall suspension of *Bacillus*. (A) Control, (B) Intact cells and (C) Cell wall suspension.

There were no detectable differences in *BRI1* expression pattern or level between control plants (Figure 9A) and plants treated with intact cells (Figure 9B). *BRI1* expression was shown to differ in roots treated with cell wall suspension (Figure 9C) compared to roots treated with intact cells and controls. Root tips of plants

treated with cell wall suspension had a high expression level during the whole time-course whereas *BRI1* expression was absent in the primary roots. The *BRI1* expression in leaves was generally the same for the two treatments and control.

4.3.3 Immunoprecipitation of *BRI1:GFP* after *Bacillus* treatment

BRI1:GFP plants were root treated with *B. amyloliquefaciens* UCMB5113 in order to investigate if BRI1 interacts with any other proteins during *Bacillus* treatment. Control plants were treated with distilled water, both control and *Bacillus* treated roots were collected two weeks post-treatment. The precipitated proteins were separated by SDS-PAGE which was visualized via western blotting using GFP-specific antibodies.

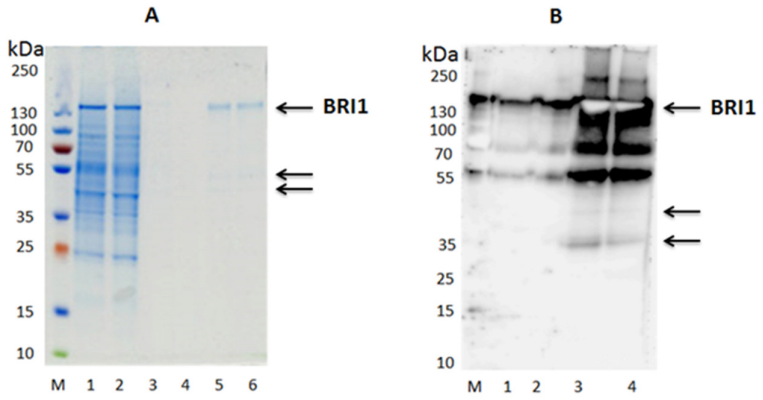

Figure 10. SDS-PAGE and Western blot analysis of precipitated *BRI1:GFP* in *Bacillus* treated plants. (A) SDS-PAGE; M. Marker, 1. Crude control, 2. Crude treated, 3. Wash control, 4. Wash treated, 5. IP control, 6. IP treated. (B) Western blot; M. Marker, 1. IP control, 2. IP treated, 3. Crude control, 4. Crude treated.

Figure 10A shows the SDS-PAGE gel where precipitated protein complexes are separated. No loss of BRI1:GFP proteins was observed after washing the pellet (lane 3-4). The immunoprecipitated proteins are shown in lane 5 (control) and lane 6 (*Bacillus* treated). The upper arrow shows precipitated BRI1 and the two lower arrows shows putative candidate ligands binding to the immunoprecipitated *BRI1:GFP* protein complex. Figure 10B shows the Western blot membrane with the transferred proteins from the SDS-PAGE gel. This experiment did not pick up any proteins interacting with *BRI1:GFP* upon *Bacillus* treatment.

4.4 The role of *ML* gene family members in *Bacillus* mediated priming of plant defense

4.4.1 qPCR analysis of *ML* genes

A gene expression analysis was performed in order to examine the expression levels of *ML* genes during root colonization of *B. amyloliquefaciens* UCMB5113. A qPCR analysis was performed with leaves of *Bacillus* treated *A. thaliana* wild type (Col-0) plants. Control plants were treated with distilled water.

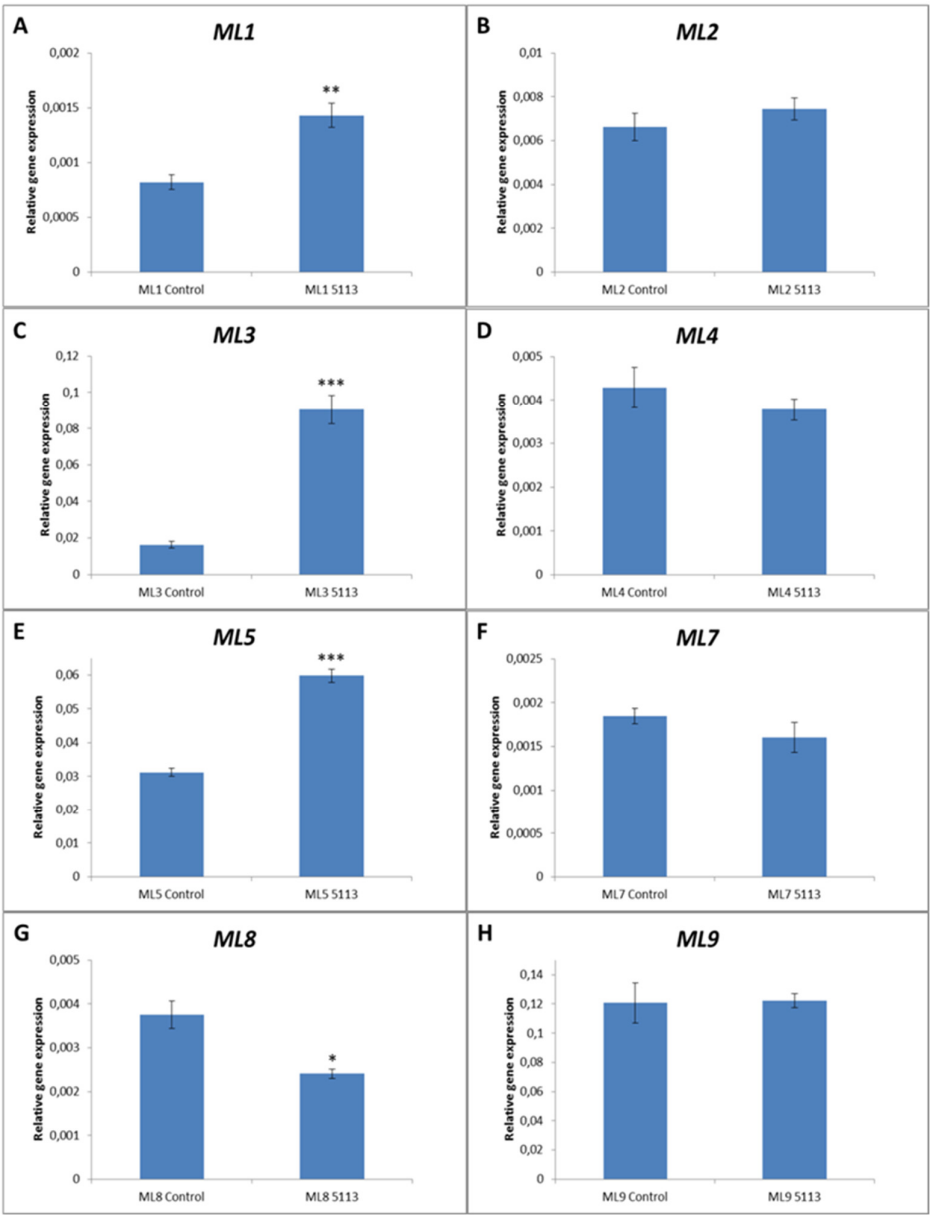

Figure 11. Expression of *ML* genes in *Bacillus* treated *A. thaliana* Col-0 plants. Significance level of data: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The results obtained from the gene expression analysis (Figure 11) showed that there was a significant difference in gene expression levels of *ML1*, *ML3*, *ML5* and *ML8* in leaves of *Bacillus* treated Col-0 plants compared to leaves of control. The expression of *ML1* in *Bacillus* treated plants showed a 1.7-fold up-regulation compared to control plants (Figure 11A). The expression of *ML3* in *Bacillus* treated

plants showed a 5.6-fold up-regulation compared to control plants (Figure 11C). The expression of *ML5* in *Bacillus* treated plants showed a 1.9-fold up-regulation compared to control (Figure 11E). The expression level of *ML8* was down-regulated in *Bacillus* treated plants compared to control plants (Figure 11G). There was no significant difference observed in the expression levels of *ML2*, *ML4*, *ML7* and *ML9* (Figure 11B/D/F/H) between the *Bacillus* treated and control Col-0 plants.

4.4.2 Efficiency of *Bacillus* treatment on *ml3-1* and *ml7-1* mutants upon *A. brassicicola* inoculation

In order to examine the role of *ML3* and *ML7* under *B. amyloliquefaciens* UCMB5113 mediated priming of defense response, mutant lines *ml3-1* and *ml7-1* were challenged with *A. brassicicola* under *Bacillus* treatment and control (distilled water) conditions. One week post-treatment with *Bacillus*, leaves of mutant lines and Col-0 were inoculated with *A. brassicicola*. The disease development was visually recorded after five days post-inoculation.

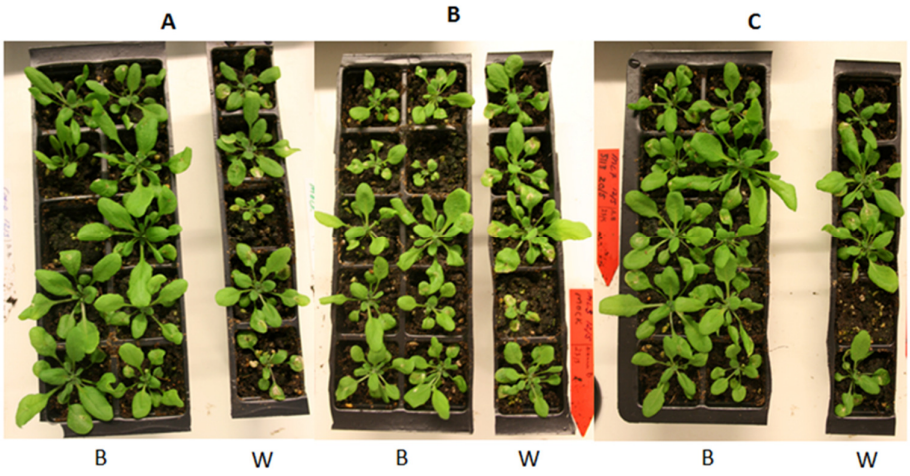

Figure 12. Disease development five days after inoculation with *A. brassicicola*. B denotes *Bacillus* treated plants and W denotes water treated plants. (A) Col-0 plants, (B) *ml3-1* plants and (C) *ml7-1* plants.

The control leaves had more necrotic lesions in comparison to leaves of *Bacillus* treated Col-0 and mutant lines (*ml3-1* and *ml7-1*) (Figure 12). The disease development of *A. brassicicola* seems therefore to be suppressed by *B. amyloliquefaciens* UCMB5113. No difference was visually recorded between primed and control condition of leaves belonging to wild type and mutant lines.

4.4.3 A time-course study on *ML3p:GUS* plants after *Bacillus* treatment and challenge inoculation with *A. brassicicola*

A GUS reporter gene assay was carried out in order to study *ML3* expression in pathogen challenged plants under *Bacillus* treatment and control conditions (distilled water). Leaves were inoculated with *A. brassicicola* two days post-treatment. The inoculated and non-inoculated leaves were collected and stained two days post-inoculation.

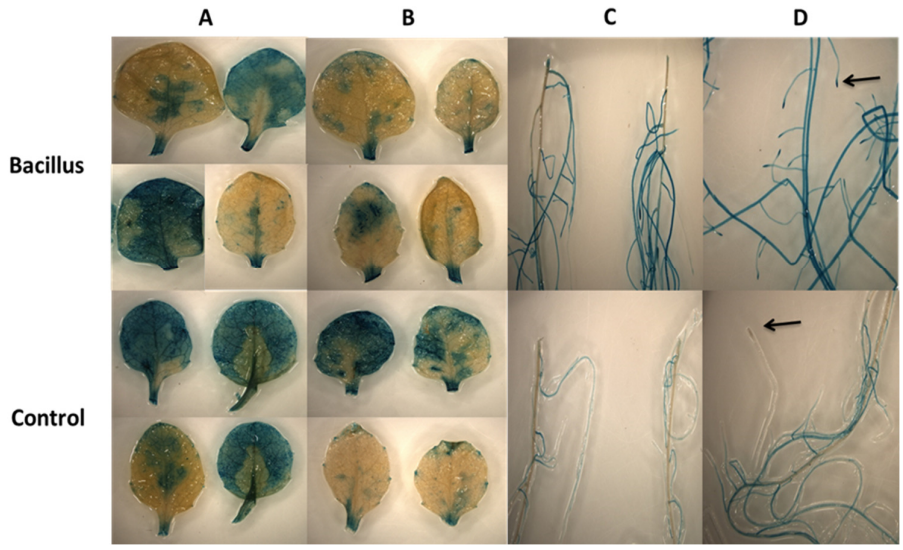

Figure 13. Two days after *A. brassicicola* inoculation on *Bacillus* treated and control *ML3p:GUS* plants. Top half of figure shows *Bacillus* treated plants and bottom half shows control plants. (A) Inoculated leaves, (B) Non-inoculated leaves, (C) Roots of inoculated plants and (D) Roots of non-inoculated plants.

Inoculated leaves of *Bacillus* treated plants showed a varying expression level and pattern of *ML3* (top half, Figure 13A). *ML3* was generally shown to be expressed at a high level in pathogen inoculated leaves of control plants (bottom half, Figure 13A). Some inoculated leaves of control plants had visible necrotic tissue as the figure shows. The non-inoculated leaves of *Bacillus* treated plants had overall low expression level of *ML3* compared to non-inoculated plants (top half, Figure 13B). The expression of *ML3* was observed to vary largely between the leaves of non-inoculated leaves of control plants (bottom half, Figure 13B). In general, *ML3* had a higher expression in roots of *Bacillus* treated plants (top half, Figure 13C/D) than in roots of control plants (bottom half, (Figure 13C/D). *ML3* was shown to be highly expressed in *Bacillus* treated root tips of non-inoculated plants whereas no expression of *ML3* was recorded in root tips of non-inoculated control plants.

An additional GUS reporter gene assay was carried out in order to study how *ML3* is expressed in plants treated with intact and cell wall *B. amyloliquefaciens* UCMB5113 suspension. This was done to assess potential expression differences of *ML3* between plants treated with intact cells and cell wall suspension. Plants were collected and stained at three time points (24 h, 4 days and 8 days) post-treatment. Control plants were treated with distilled water.

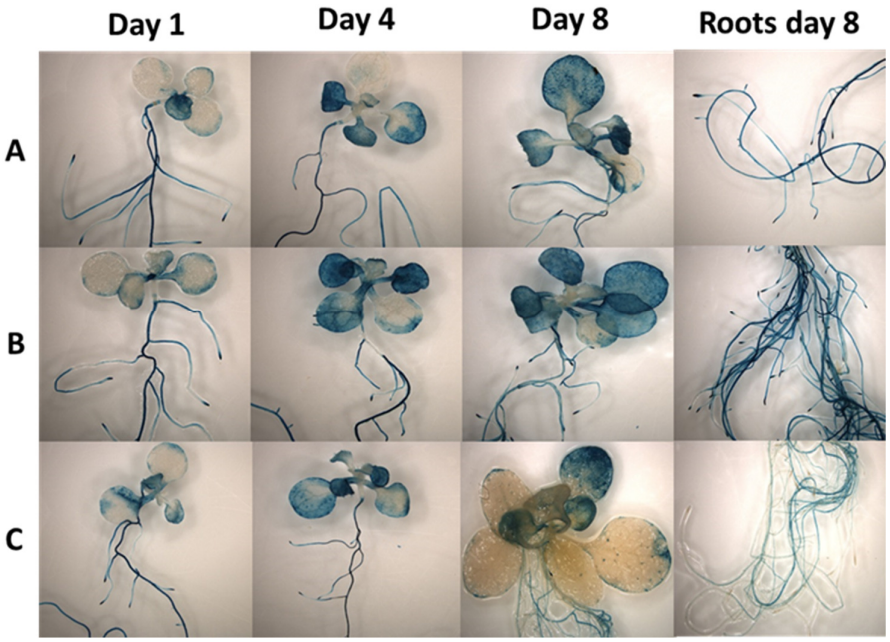

Figure 14. A time-course study on *ML3p:GUS* plants treated with intact cell suspension and cell wall suspension of *Bacillus*. (A) Control, (B) Intact cells and (C) Cell wall suspension.

There were no visual differences in *ML3* expression pattern or level between control plants (Figure 14A) and plants treated with intact cells (Figure 14B). For those two treatments, the expression pattern of *ML3* increased from day 1 to day 8. *ML3* was highly expressed in roots at day 8 for control plants and plants treated with intact cells (Figure 14A/B). The observed *ML3* expression pattern or level did not differ largely from plants treated with cell wall suspension (Figure 14C). However, the *ML3* expression was slightly lower in leaves and root at day 8 compared to control plants and plants treated with intact cells (Figure 14C). Interestingly, no expression of *ML3* was observed in roots tips of plants treated with cell wall suspension at day 8 time interval.

5 Discussion

5.1 The role of BRs in plant growth and stress response upon *B. amyloliquefaciens* UCMB5113 treatment

5.1.1 Effect of *Bacillus* treatment on BR deficient plants

In order to elucidate the role of BRs in *B. amyloliquefaciens* UCMB5113 mediated priming of plant growth and defense response, a phenotypic study was carried out in control and *Bacillus* treated plants (wild type Col-0 and BR deficient *det2-1*). The results showed a clear distinction in root growth of *Bacillus* treated and control plants after eight and 12 days post-treatment. The *Bacillus* treated wild type plants had more lateral roots and somewhat larger leaves than the control plants. The results are in line with a previous study, which observed an increased root biomass in oilseed rape treated with *B. amyloliquefaciens* UCMB5113 (Bejai *et al*., 2009). This phenotypic distinction was also observed for *Bacillus* treated *det2-1* plants, which also had more lateral roots and larger leaves. It has earlier been shown that the dwarfed mutant phenotype of *det2-1* can be restored by exogenous brassinolide application (Fujioka *et al*., 1997). A possible explanation for the primed growth promotion could therefore be that *B. amyloliquefaciens* UCMB5113 affects the expression of BR associated genes via a released molecule like brassinolide or another BR mimic. This molecule could potentially initiate BR signaling by binding to BRI1 during root colonization which enables growth promotion even in BR deficient plants.

Further, no notable difference was observed between the flowering phenotype of *Bacillus* treated and control wild type plants three weeks post-treatment in soil. However, the phenotype of treated *det2-1* plants was distinct with higher plants compared to the control *det2-1* and similar to wildtype plants. This could also depend on release of some BR like molecule from *B. amyloliquefaciens*

UCMB5113 during priming which potentially induces BR signaling as discussed previously.

Together, the results demonstrate that root treatment with *B. amyloliquefaciens* UCMB5113 resulted in growth promotion in wild type (Col-0) and BR deficient (*det2-1*) plants. Therefore, it seems like BRs are involved in the root colonizing and priming events leading to enhanced growth. Stimulated root growth, as observed here, can benefit plants in several ways by e.g. increasing water and nutrient acquisition from soil (Péret *et al*., 2009).

Unfortunately, no results were obtained from the BR analysis due to technical problems with instruments. Therefore, no data are shown regarding what BRs that are present and the endogenous levels of them in Col-0 and *det2-1* plants under primed and non-primed conditions. An experimental repeat with BR analysis is therefore suggested in order to gain better understanding about the role of BRs in priming mediated by *B. amyloliquefaciens* UCMB5113. This would demonstrate how the endogenous BR profile and levels are affected by treating roots of Col-0 and *det2-1* with *B. amyloliquefaciens* UCMB5113.

5.1.2 Effect of *Bacillus* treatment upon pathogen challenge in BR deficient plants

Control and treated plants (Col-0 and *det2-1*) were challenged with *A. brassicicola* in order to examine the role of BRs for primed defense responses mediated by *B. amyloliquefaciens* UCMB5113. The necrotic lesions observed were larger in control Col-0 plants than in *Bacillus* treated Col-0 plants. This observation indicates that *B. amyloliquefaciens* UCMB5113 is able to mediate primed defense responses against *A. brassicicola* in wild type plants. The observed result is consistent with an earlier study which showed that *B. amyloliquefaciens* UCMB5113 was able to provide protection against *A. brassicicola* in oilseed rape (Danielsson *et al*., 2006). ISR is known to provide protection against *A. brassicicola* in *A. thaliana* plants (Ton *et al*., 2002). The protective effects against *A. brassicicola* by plant treatment with *B. amyloliquefaciens* UCMB5113 can therefore probably explained by the ability of this strain to prime ISR similar to other rhizobacteria like some Pseudomonas strains (Van Loon, *et al*., 1998).

Two control *det2-1* plants were not viable after *A. brassicicola* challenge. The viable *det2-1* control had visible necrotic lesions. In contrast, all *Bacillus* treated *det2-1* plants were viable after the inoculation. However, no reduction of necrotic lesions was observed in *Bacillus* treated *det2-1* plants. From this comparison, it can be assumed that *B. amyloliquefaciens* UCMB5113 is partly able to mediate defense response against *A. brassicicola* in *det2-1* mutants. It is also possible that

the improved growth of the *Bacillus* treated *det2-1* mutants influenced the results. A larger rosette size could potentially be more robust when exposed to challenge. BRs could potentially be involved in defense responses since the results showed that challenged control *det2-1* mutants appeared to be less resistant against *A. brassicicola* than *Bacillus* treated *det2-1* mutants. It is possible, as previously discussed, that *B. amyloliquefaciens* UCMB5113 restores the BR deficient phenotype by releasing some BR like molecule, which potentially serves in BR signaling leading to enhanced defense capabilities. This suggestion could potentially be supported by an earlier study where disease resistance was shown to be induced against several pathogens by brassinolide treatment of tobacco and rice (Nakashita *et al*., 2003). It would therefore be interesting in future research to exogenously treat *det2-1* mutants with brassinolide to restore the phenotype to more wild type appearance and challenge rescued plants with *A. brassicicola*. A comparison of the disease development in restored challenged *det2-1* mutants and controls is suggested to be done in order to assess whether the lack of BR has a detrimental role in plants ability to initiate defense responses upon pathogen stress or not.

Assessing the ability of *B. amyloliquefaciens* UCMB5113 to mediate primed defense responses against *A. brassicicola* was done by quantifying *A. brassicicola* DNA relative to the *A. thaliana* DNA. There was a significant difference in *AbrCUT* expression between control Col-0 leaves and *Bacillus* treated Col-0 leaves. The qPCR analysis showed an 8-fold up-regulation of *AbrCUT* in Col-0 control leaves in comparison to leaves of *Bacillus* treated Col-0 plants. This demonstrates that *A. brassicicola* was more abundant under control condition compared to the primed condition. The result shows that *B. amyloliquefaciens* UCMB5113 is able to mediate primed defense responses in Col-0 plants challenged to *A. brassicicola*. The quantification results are consistent with the results obtained from the phenotypic study where disease development was visually suppressed in challenged *Bacillus* treated Col-0 plants. The result is in agreement with Danielsson *et al*. (2006) which, as mentioned, showed that *B. amyloliquefaciens* UCMB5113 could provide protection against *A. brassicicola* in oilseed rape. However, a large standard deviation was observed for the qPCR data of Col-0 control which gives the significance some uncertainty. Therefore, the pathogen quantification needs to be repeated to have more robust data.

No significant difference was observed between the relative expression of *AbrCUT* in *det2-1* under control conditions and *det2-1* under primed conditions when challenged with *A. brassicicola*. This was slightly contrary to the phenotypic observation where some visible protection was recorded when comparing *Bacillus* treated and control *det2-1* plants. But as earlier discussed, the control *det2-1* plants

were substantially smaller which could have interfered with the result. However, the current results show that *B. amyloliquefaciens* UCMB5113 is unable to mediate primed defense responses against *A. brassicicola* in *det2-1* mutants. Sufficient BR levels are potentially crucial for the defense response since BR is known to be involved in the innate immunity of plants (Chinchilla *et al*., 2007; Nakashita *et al*., 2003). It may be that *B. amyloliquefaciens* UCMB5113 does not increase BR levels above some critical threshold level to provide protection against pathogens like *A. brassicicola*. This could further be investigated by comparing the BR levels in challenged control and primed plants (Col-0 and *det2-1*) in a repeated BR analysis as earlier proposed.

5.2 The role of BR receptors BAK1 and BRI1 during *B. amyloliquefaciens* UCMB5113 colonization of *A. thaliana* roots and enhanced plant defense

The role of BR receptors (BAK1 and BRI1) in primed defense responses against *A. brassicicola* was investigated in wild type Col-0 and mutant lines *bak1-4* and *bri1-1*. When comparing the disease development between wild type and mutant lines, no visual difference was observed between *Bacillus* treated plants and the respective controls. The leaves of wild type and mutant lines had in general similar amounts of necrotic lesions. It is therefore difficult to visually predict the role of BAK1 and BRI1 under performed conditions when challenged to *A. brassicicola*. The absence of primed defense response in wild type was unexpected since a reduced disease development was observed in the previous pathogen assay for *Bacillus* treated wild type.

However, the disease development observed in control *bak1-4* plants was somewhat expected since BAK1 is known to participate in plant immunity processes (Chinchilla *et al*., 2007). Potentially, when BAK1 is absent it cannot interact with FLS2 known to induce immunity processes when recognizing bacterial flagellin proteins. The inability of *B. amyloliquefaciens* UCMB5113 to mediate primed defense responses in *bak1-4* plants indicates a possible non-recognition factor, which disables ISR activation in the mutant plants. It has earlier been shown that *bak1-4* mutants develop an increased amount of necrotic lesions in comparison to Col-0 when challenged with *A. brassicicola* (Kemmerling *et al*., 2007). The same study demonstrates that the plant immunity involvement of BAK1 is independent of its role in BR signaling. Therefore, disease protection should be present in the *bri1-1* mutants since BAK1 can initiate defense signaling independently without complexing with BRI1. This does not appear to be the case here since the same disease development was observed as mentioned. The experiment needs to be

repeated including quantification of pathogen levels by qPCR in order to establish more reliable data about the role of BAK1 and BRI1 in *B. amyloliquefaciens* UCMB5113 mediated priming of defense responses.

5.3 Identification of a potential MAMP of *B. amyloliquefaciens* triggering BR signaling

5.3.1 A time-course study on *BAK1:GUS* plants after treatment with *Bacillus* and challenge inoculation with *A. brassicicola*

In order to study *BAK1* expression in plants treated with *B. amyloliquefaciens* UCMB5113, a GUS reporter gene assay was done, collecting plants 1-5 days post-treatment. *BAK1* expression was absent in controls during the whole time-course indicating that *BAK1* expression is induced when roots are treated with *B. amyloliquefaciens* UCMB5113. The observed result shows that *BAK1* was expressed over the whole rosette at day 1. The expression was shown to increase at day 2 which remained at the same level to day 5. The lower expression at day 1 could potentially be explained by that *BAK1* expression increases gradually as the bacterial root colonization increases. Alternatively, the lower *BAK1* expression at day 1 could relate to the ability of beneficial bacteria to modulate the MAMP triggered defense responses to colonize properly (Zamioudis and Pieterse, 2012). Such a scenario is possible since BAK1 is known to be involved in MAMP triggered defense responses (Chinchilla *et al*., 2007).

Further, the expression pattern indicates that *BAK1* expression is not limited to the root area where the bacterial treatment was performed. The current study shows that *BAK1* is systemically expressed in leaves when the root is treated with *B. amyloliquefaciens* UCMB5113. Locally induced *BAK1* emerging systemically to leaves is potentially favorable for the plant since it could provide the plant with an increased protection against pathogens aboveground. During the whole time-course, the expression levels were lower in roots compared to leaves which might depend on that leaves are more exposed aboveground in contrast to roots in soil. Also, *BAK1* was shown to be highly expressed in root tips of secondary roots suggesting that the expression of *BAK1* is elevated in the root tip area as the plant recognizes *B. amyloliquefaciens* UCMB5113 during colonization.

The expression of *BAK1* was further studied in *Bacillus* treated plants challenged by *A. brassicicola* using an additional GUS assay. No expression of *BAK1* was observed in control plants indicating that *BAK1* is expressed in *Bacillus* treated plants under both inoculated and non-inoculated conditions. However, the expres-

sion pattern of *BAK1* was shown to differ significantly when comparing the different conditions. In non-inoculated *Bacillus* treated plants, the expression pattern was located at the apex and margin of the leaves. The expression of *BAK1* was shown to relocate to the center of the leaf when the plant was inoculated. The observed change in expression of *BAK1* can most likely be linked to its involvement in primed defense responses since the centric pattern corresponds to the area where the inoculation was performed. This indicates that *BAK1* responds to the exposure of *A. brassicicola* since the expression is directed to the site of infection. A possible explanation for this result is that BAK1 potentially recognizes some MAMP associated with *B. amyloliquefaciens* UCMB5113 during colonization. Such recognition leads to an early induction of ISR which systemically primes the plant for faster and a more robust defense upon attack as potentially seen in the results (Van der Ent *et al*., 2009). Other possible explanations are that BAK1 potentially recognizes some damage-associated molecular patterns (DAMPs) released from the damaged tissue caused by *A. brassicicola* or that *BAK1* gets upregulated as DAMPs are recognized by e.g. Toll-like receptors causing activation of the innate immunity (Lotze *et al*., 2007).

5.3.2 A time-course study on *BRI1:GUS* plants after pre-treatment with *Bacillus* cell wall suspension

BRI1 expression was studied in plants treated with *B. amyloliquefaciens* UCMB5113 by performing a GUS reporter gene assay collecting plants 1-3 days post-treatment. A suspension of *B. amyloliquefaciens* UCMB5113 cell walls was used in this assay to compare potential expression differences of *BRI1* in plants treated with intact cells. The result showed that there were no detectable differences in *BRI1* expression pattern or level between plants treated with intact cells and controls. Interestingly, the expression of *BAK1* in plants treated with cell wall suspension was shown to be restricted to secondary roots and absent in the primary roots. The result demonstrates that the bacteria do not have to be alive to trigger *BRI1* expression. A possible explanation for this is that there may be some surface molecule associated with the cell wall responsible for the induction of *BRI1* expression upon root treatment.

Taken together, the observed expression pattern suggests that *BRI1* expression responds differentially depending on what condition the bacterial cells are in when used for treatment. It would be interesting to investigate whether treating roots with cell wall suspension would result in growth promotion as earlier observed for intact bacterial cells. In addition, since BAK1 is the co-receptor of BRI1, it would be interestingly to repeat the preformed assay by treating *BAK1:GUS* plants with

cell wall suspension to assess whether cell wall suspension also triggers *BAK1* expression.

5.3.3 Immunoprecipitation of *BRI1:GFP* after *Bacillus* treatment

Immunoprecipitation of *BRI1:GFP* was performed in order to detect whether BRI1 perceives any molecule released by *B. amyloliquefaciens* UCMB5113 during root treatment. According to the results obtained from SDS-PAGE and western blotting, it was shown that BRI1 was precipitated together with some protein binding to it during both control and primed conditions. However, no additional bands were observed in the *Bacillus* treated samples, this could be inferred that the new potential ligands might be present but are of low mass and are therefore not able to be seen in this SDS-PAGE. An additional experiment is suggested where the precipitated proteins would be detected by a high-resolution polyacrylamide gel electrophoresis in order to reveal new potential ligands of low mass. In addition, since the presented GUS assay demonstrated that the expression of *BAK1* responds to the exposure to *B. amyloliquefaciens* UCMB5113, it would be interesting to perform an additional immunoprecipitation including *BAK1:GFP* plants to assess whether BAK1 perceives a molecule during *Bacillus* treatment.

5.4 The role of *ML* gene family in *Bacillus* mediated priming of plant defense

5.4.1 qPCR analysis of *ML* genes

A gene expression analysis was performed to examine the expression levels of *ML* genes during root colonization of *B. amyloliquefaciens* UCMB5113. The results revealed that there was a significant difference in gene expression of *ML1*, *ML3*, *ML5* and *ML8* in leaves of *Bacillus* treated Col-0 plants compared to leaves of control Col-0 plants. *ML1*, *ML3*, *ML5* was shown to be up-regulated in response to *B. amyloliquefaciens* UCMB5113 whereas *ML8* was shown to be down-regulated in comparison to the control. *ML3* was expressed at a higher level compared to the other tested *ML* genes. The gene expression analysis indicates a probable role for *ML* genes (1,3,5,8) during the colonization process of *B. amyloliquefaciens* UCMB5113. The expression results are in line with a previous study which showed that *ML5* and *ML9* were up-regulated in *A. thaliana* roots after treatment with the biocontrol bacterium *Pseudomonas fluorescens* (Van de Mortel *et al*., 2012). A recent study has identified *ML3* as a potential participant in JA signaling mediated defense responses (Fridborg *et al*., 2013; Hakenjos *et al*., 2013). The observed up-regulation of *ML* genes may therefore be linked to the ability of *B. amyloliquefaciens* UCMB5113 in activating ISR via JA signaling.

5.4.2 Efficiency of *Bacillus* treatment on *ml3-1* and *ml7-1* mutants upon *A. brassicicola* inoculation

In order to understand the role of *ML3* and *ML7* under *B. amyloliquefaciens* UCMB5113 mediated priming of defense responses, the mutant lines *ml3-1* and *ml7-1* were challenged with *A. brassicicola* under primed and non-primed conditions. The visual recordings revealed that controls of Col-0 and mutant lines (*ml3-1* and *ml7-1*) had more necrotic lesions compared to *Bacillus* treated plants. It therefore seems like *B. amyloliquefaciens* UCMB5113 is able to mediate disease suppression in all tested lines independently of the mutations present. The observations also suggest that *ML3* and *ML7* do not have any major role in ISR since there was no difference observed between the disease phenotype in control *ml3-1* and *ml7-1* compared to Col-0 controls. It would be interesting to repeat the pathogen assay using *ml3-3* instead since Hakenjos *et al*. (2013) identified it as a good mutant candidate with no ML3 protein present.

Additionally, generating single knockout mutants might be insufficient since it has been demonstrated that some *ML* gene members group together due to high sequence similarity upon phylogenetic analysis (Fridborg *et al*., 2013). Therefore, generating multiple knockout mutants or RNA suppression might be useful in future research to be able to receive more distinguishable results and suppress redundancy effects.

5.4.3 A time-course study on *ML3p:GUS* plants after priming with *Bacillus* and challenge inoculation with *A. brassicicola*

In order to study *ML3* expression in *Bacillus* treated plants challenged with *A. brassicicola*, a GUS reporter gene assay was carried out collecting plants 2 days after treatment. The observation revealed that *ML3* expression level and pattern varied largely in leaves of inoculated *Bacillus* treated plants. *ML3* was shown to be expressed at a high level in inoculated leaves of all control plants. Necrotic lesions were visible in some of the inoculated leaves of control plants. The expression of *ML3* was low in leaves of non-inoculated *Bacillus* treated plants in comparison to the high *ML3* expression observed in leaves of control non-inoculated plants. *ML3* was highly expressed in the roots of *Bacillus* treated plants under inoculated and non-inoculated conditions. The expression of *ML3* was significantly lower in roots, especially in the tips, of control plants, however, no difference was observed in the inoculated and non-inoculated plants.

The results clearly show that the expression of *ML3* is elevated in roots upon colonization of *B. amyloliquefaciens* UCMB5113. This result is in line with the expression analysis which revealed an up-regulation of *ML3* in response to *B. amylo-*

liquefaciens UCMB5113. However, it is difficult to predict the role of *ML3* in *Bacillus* treated defense responses from the current observations since the expression level and pattern varied largely. The expression of *ML3* was shown to vary between the leaves of the non-inoculated controls where *ML3* was highly expressed in some leaves and almost absent in others. The expression was overall low in all *Bacillus* treated controls. The highly expressed *ML3* in non-inoculated controls might be explained by the role of *ML3* in early development stages of *A. thaliana* (Fridborg *et al*., 2013).

However, *B. amyloliquefaciens* UCMB5113 seems to be able to promote defense response since no necrotic lesions were observed in leaves of treated inoculated plants compared to controls. It is difficult to say whether the promoted defense response is independent of *ML3* or not since *ML3* expression was almost absent in some of the inoculated leaves of *Bacillus* treated plants. Potentially, there may be a relationship between the absence of *ML3* in inoculated leaves of *Bacillus* treated plants and elevated levels of *ML3* in their roots. These results show that *Bacillus* treated roots lead to an up-regulation of *ML3* which further induces JA signaling, in turn activating ISR when the plant is challenged with a necrotrophic pathogen. Such activation, originating from elevated *ML3* expression in roots, might provide the plant with a systemic protection in aboveground tissues and not just in the root area where *ML3* was shown to be expressed. The experiment needs to be repeated in order to establish the role of *ML3* in primed defense responses since earlier findings have suggested the involvement of *ML3* in plant immune response (Fridborg *et al*., 2013; Hakenjos *et al*., 2013).

Finally, an additional GUS assay was done in order to study how *ML3* is expressed in plants treated with intact and a cell wall suspension of *B. amyloliquefaciens* UCMB5113. Plants were collected and stained at three time points (24 h, 4 days and 8 days) post-treatment. In general, no difference was observed between the expression levels of *ML3* between control and plants treated with intact cell suspension. Therefore, it is difficult to predict how *ML3* is affected when treated with *B. amyloliquefaciens* UCMB5113 from this observation. Interestingly, *ML3* was less expressed in plants treated with cell wall suspension. The expression of *ML3* was completely absent in the root tips in plants treated with cell wall suspension. The previous GUS assay demonstrated that *ML3* expression was absent in root tips of control plants in comparison to treated roots where it was highly expressed. The visual recordings suggest that viable bacteria are required for triggering *ML3* expression. A qPCR analysis could be performed in order to quantify the expression levels of *ML3* in roots treated with intact cells respective cell wall suspension to confirm the histochemical observation.

6 Conclusion

This study confirms that *B. amyloliquefaciens* UCMB5113 is capable of promoting growth in wild type Col-0 compared to controls. The investigations revealed that *B. amyloliquefaciens* UCMB5113 also mediate enhanced growth in the BR deficient mutant *det2-1*. *B. amyloliquefaciens* UCMB5113 was therefore suggested to be able to promote plant growth by elevating the endogenous BR levels. It was proposed that *B. amyloliquefaciens* UCMB5113 mediate enhanced growth by releasing some molecule that can bind to BRI1 and priming would involve BR signaling. Pathogen assays showed that *B. amyloliquefaciens* UCMB5113 was able to mediate primed defense responses in wild type Col-0 but not in *det2-1* mutants. The GUS assays showed that *BAK1* was systemically expressed in leaves under primed conditions. *BAK1* expression was shown to relocate to inoculation site upon challenge, suggesting a role for BAK1 both in colonization and defense responses. *BRI1* expression was also induced during root colonization by *B. amyloliquefaciens* UCMB5113. Interestingly, *BRI1* was shown to be induced by a cell wall suspension of *B. amyloliquefaciens* UCMB5113 revealing that the non-viable bacteria can trigger *BRI1* expression. It is still unknown whether non-viable bacteria can promote growth and defense, but this could be assessed in future research. Gene expression analysis indicated a probable role for *ML* genes (1,3,5,8) during the colonization process of *B. amyloliquefaciens* UCMB5113. However, the preliminary studies performed on *ml3-1* and *ml7-1* mutants do not indicate that they have any major role in ISR. Preliminary studies suggest an involvement of BRs during *B. amyloliquefaciens* UCMB5113 mediated plant growth and defense, however more research is suggested to define the mode of action. Finally, future work is proposed to continue investigating the mechanisms behind *B. amyloliquefaciens* UCMB5113 mediated priming and whether a surface molecule associated with *B. amyloliquefaciens* UCMB5113 is responsible for or contributes to primed growth and defense responses.

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