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Abstract

It is of interest to gain knowledge of the mechanisms operating behind priming, an important possible strategy to help plants handle stress. The hypothesis is that priming of *Arabidopsis thaliana*, as a result of colonization of roots using the beneficial bacteria *Bacillus amyloliquefaciens*, stimulates Brassinosteroid (BR) synthesis in plants leading to plant growth promotion and improved defense responses. The beneficial bacterium *B. amyloliquefaciens* (strain UCMB5113) was used for priming of wild type Col-0 and different mutant lines of *A. thaliana* in order to study the gene expression of different BR-related genes using real-time PCR (qPCR). The BR-related genes *DET2, BAK1, BRI1* and *DWF1* were found to be involved in priming. Gene expression of *BAK1*, *BRI1* and *MPK4* was examined during *Bacillus* mediated priming and the result showed that *Bacillus* is likely to bind to BAK1 during priming. It was of interest to see whether a surface molecule on *Bacillus* could be responsible for priming. This was examined using heat killed *Bacilli* with focus on effects on *BAK1* expression, compared with regular priming. The result showed that it is likely that a surface molecule on *Bacillus* is responsible for activation of *BAK1* upon priming. Effects of herbivore and pathogen attack on *A. thaliana* during priming were also investigated using larvae of *Spodoptera littoralis* and *Pseudomonas syringae* pv. tomato strain DC3000, respectively. Both treatments indicated that the BR-related gene *BAK1* is of great importance in defense responses to pathogen and herbivore attack. It was shown that primed plants were much more tolerant to different kinds of stress than control plants and could protect the plants to a certain degree from herbivory or disease.

Sammanfattning

Det är av intresse att förstå olika mekanismer inom begreppet ”priming” då detta kan vara en viktig strategi för att hjälpa växter hantera stress. Hypotesen är att priming av *Arabidopsis thaliana* genom kolonisering av växtens rötter med bakterien *Bacillus amyloliquefaciens*, kommer stimulera syntes av Brassinosteroider (BR), vilket leder till ökad tillväxt och förbättrat försvar. Bakterien *B. amyloliquefaciens* (stam UCMB5113) användes vid priming av Col-0 och olika mutanta linjer av *A. thaliana* för att kunna studera genuttrycket av olika BR-relaterade gener med kvantitativ realtids-PCR (qPCR). Experimentet indikerade att de BR-relaterade generna *DET2, BAK1, BRI1* och *DWF1* är viktiga vid priming. Genuttryck av *BAK1*, *BRI1* och *MPK4* undersöktes och resultaten pekar på att *Bacillus* troligen binder till BAK1 vid priming. För att förstå om mekanismen bakom priming beror på en ytmolekyl på *Bacillus* användes värmeavdödade bakterier med fokus lagd på genuttrycket av *BAK1*. Detta genuttryck för *BAK1* jämfördes med vanlig priming och resultatet visade att det är troligt att en ytmolekyl på *Bacillus* är ansvarig för aktivering av *BAK1* vid priming. Effekterna av insekt- och patogenangrepp på *A. thaliana* efter priming undersöktes med hjälp av både *Spodoptera littoralis* och *Pseudomonas syringae* pv. tomat DC3000. Resultaten indikerade att den BR-relaterade genen *BAK1* var av stor vikt för växtens försvar mot insekter och patogener. Det visade sig att de växter som utsatts för priming var mycket mer toleranta mot olika typer av stress och hade ett visst skydd mot insekter och sjukdom, detta i jämförelse med kontrollväxter.

Content

1. Introduction

1.1 Priming

Soil is an environment full of life, where a wide diversity of microorganisms can be found. Many of these microorganisms can have species specific interactions with plants and even have beneficial effects on the plants. Beneficial bacteria can protect plants from pathogens, promote plant growth or simply have a beneficial health effect (Danielsson *et al*. 2007) in a process referred to as priming. Priming results in a stronger and faster defense response when the plant is stressed; which is a promising approach to protect plants from both abiotic and biotic stress (Conrath *et al*. 2006; Van der Ent *et al*. 2009). In this study, priming was performed on the plant *Arabidopsis thaliana* using *Bacillus amyloliquefaciens,* which is a rhizosphere colonizing, spore forming bacterium with an orange pigment (Bejai *et al*. 2009). Strains of *Bacillus* have shown to be useful when working with bacteria in laboratory environments since they are easy to cultivate and can be stored for a long time period (Reva *et al*. 2004).

1.2 Plants

The plants that were used in the experiments performed were wild type Columbia (Col-0) and mutant lines of *A. thaliana*. The different mutant lines that were used are *det2* (defective in BR accumulation) and *bak1-4* (defective in BR perception due to receptor malfunction).

1.3 Pathogens

First instar larvae of *Spodoptera littoralis* and *Pseudomonas syringae* pv. tomato strain DC3000 were used to challenge the plants in the experiments. The insect *S. littoralis* has a very broad host range and is for that reason a problem for agriculture in many countries (Abdel-Rahman *et al*. 2007). *P. syringae* is a gram-negative bacterium that can infect a diversity of different plants. The pathogen is often able to infect the plant through openings or wounds in leaves (Katagiri *et al*. 2002). *P. syringae* is an effective pathogen since it has certain virulence factors (effectors) that are able to subdue some defense responses from the host (Pieterse *et al*. 2009).

1.4 Induced systemic resistance

When the plant is forced to defend itself, the primary response after recognition of the pathogens is the hypersensitive response (HR) often resulting in localized cell death. The secondary response is induced resistance to the pathogen. There are different defense systems that can be stimulated by bacteria; systemic acquired resistance (SAR) as well as induced systemic resistance (ISR). SAR is known to be induced by biotrophic pathogens, while ISR is induced by root colonizing rhizobacteria (Nakashita *et al*. 2002; Sadava *et al*. 2011). ISR depends on jasmonic acid (JA) and ethylene (ET), in contrast to SAR which depends on salicylic acid (SA). It has been proposed that ISR is associated with a greater sensitivity to the hormones JA and ET and not to a higher production (Conrath *et al.* 2006). There is a lot of interaction, both antagonistic and synergistic between the SA, JA and ET pathways. For example, it has been shown that JA signaling can be suppressed when the SA pathway is activated by *P. syringae* (Pieterse *et al*. 2009).

The bacteria that induce ISR are for example *Pseudomonas* spp. and *Bacillus* spp. The pathways in ISR have been shown to differ slightly depending on if ISR is induced by *Pseudomonas* spp. or

Bacillus spp. where additional pathways seem to be activated by *Bacillus* spp. (Kloepper *et al*. 2004). *B. amyloliquefaciens* 5113, which was used in the experiments to prime the plants, can induce ISR. The defense will not be activated until the plant is exposed to a pathogen and at that time, the defense response will be either faster or stronger (Conrath *et al*. 2006).

Plants are able to recognize certain conserved molecular features on attackers, for example flagellin. Such structures are called MAMP (microbe-associated molecular patterns) and they are of importance in ISR (Millet *et al*. 2010). BAK1 has been shown to interact with the flagellin receptor (FLS2) and in turn regulate innate immunity in *A. thaliana* and is therefore involved in defense against pathogens (Millet *et al*. 2010; Yang *et al*. 2010; Yang *et al*. 2011). It has been shown in wild type Col-0 that *MPK4* is activated by flg22 in the defense response to MAMP recognition (Panstruga *et al.* 2009; Suarez-Rodriguez *et al.* 2007). Other genes involved in defense that were investigated were *VSP2* (vegative storage protein 2) and *PR1* (pathogenesis-related gene 1). *VSP2* in *Arabidopsis* is a JA-responsive gene induced by wounding or different types of stress (Liu *et al*. 2006). *PR1* is a gene induced during SAR and thereby also by elevated levels of SA (Lebel *et al*. 1998).

1.5 Brassinosteroids

Brassinosteroids (BRs) have been shown to be involved in different kinds of stresses. However, the mechanisms are not fully understood. Other hormones such as JA and SA are involved in the process as well (Divi *et al*. 2010). It has been shown through transcriptomic analysis that there is an up-regulation of BRs in primed plants (Bejai *et al*. 2009). BR is also involved in a variety of developmental processes such as growth, regulation of stem as well as cell elongation, growth of pollen tubes, bending- and unrolling of leaves and root inhibition (Nakashita *et al.* 2002; Sadava *et al*. 2011).

The most active BR is called Brassinolide and can be synthesized from the precursor Campesterol. There are several pathways from Campesterol where Brassinolide can be synthesized (Figure 1), the pathway of late C-6 oxidation and the pathway of early C-6 oxidation. *DET2* is involved in a reduction step of sterols in the conversion of Campesterol to Campestanol. *DWF4* is important in several steps in BR biosynthesis and involved in the synthesis of a 22-hydroxylase. *CPD* is involved in the synthesis of 6-Deoxocathasterone and Teasterone, probably by participating in a reaction before the 23α-hydroxylation reaction involving the two proteins CYP90C1 and CYP90D1 (Divi and Krishna, 2009; Guo *et al*. 2010).

The receptor for BR in BR signaling is BRI1 (BR insensitive 1), which is a plasma-membrane receptor (Bishop and Koncz, 2002; Divi *et al*. 2010). The co-receptor for BRI1 is BAK1, which also is involved in BR signaling. Upon BR binding the C-terminal domains of BRI1 are phosphorylated and an inhibitory protein is released resulting in association of BRI1 with BAK1. The exact mode of action is not known, but a cascade of phosphorylations and dephosphorylations occur. Inactivation of BIN2 (negative regulation of transcription factors BZR1 and BES1 by phosphorylation) and activation of BSU1 (positive regulation of BR signaling by dephosphorylation of transcription factors BZR1 and BES1) will occur upon BRI1 binding to BR (Figure 2). The transcription factors can regulate the expression of BR-responsive genes, either by suppression or activation (Divi *et al*. 2009; Guo *et al*. 2010). Recently it has been shown that appropriate levels of endogenous BR levels are necessary for optimal MAMP signaling (Albrecht *et al*., 2012; Belkhadir *et al*., 2012; Wang 2012).

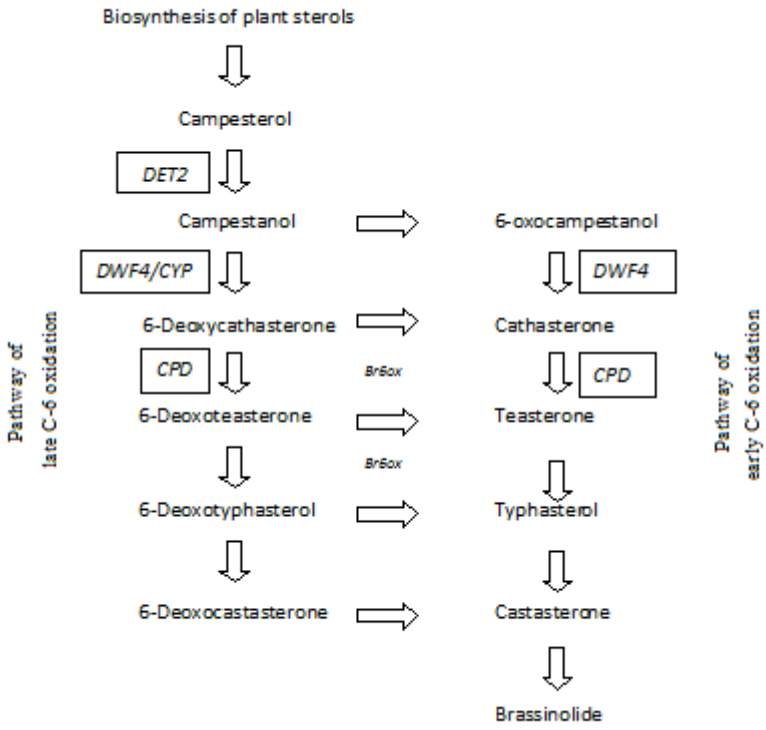

Figure 1. Pathway of BR biosynthesis.
(Modified from Divi and Krishna, 2009)

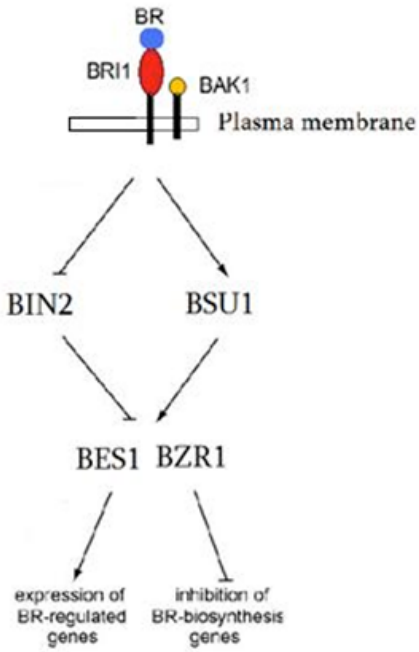

Figure 2. Model for BR signaling pathway.
(Modified from Divi and Krishna, 2009.)

2. Aims

2.1 The aim of the project

It is of interest to understand what processes that take place within the plant during priming in order to increase stress tolerance in the plant that is of prime agricultural interest. The main aim of the project was to understand the role of BRs in plants during *Bacillus* mediated priming of plants for enhanced stress tolerance. It was of interest to see how BR-related genes are affected by priming, to see if the BR receptor is necessary for colonization by *Bacillus* and if a *Bacillus* surface molecule is responsible for *Bacillus* colonization. The hypothesis is that when beneficial bacteria *B. amyloliquefaciens* are applied to roots of *A. thaliana* this increases the endogenous BR levels of plants in turn leading to plant growth promotion and improved defense responses upon challenge. To elucidate the mode of action during the recognition of *Bacillus* on the plant surface and colonization pattern leading to ISR, BR-related genes were investigated.

3. Materials and methods

3.1 Plant material

A. thaliana mutants (*bak1-4, det2*) (Chinchilla *et al*. 2007; Noguchi *et al*. 1999) and wild type Col-0 were planted on standard soil (S-jord, Hasselfors garden) and on MS media. The plants were grown under the following conditions: 22°C day and 18°C night, 16 h/8 h (day/night), a fluorescent light intensity of 200 µmol $m^{-2}s^{-2}$, 70 % RH.

3.2 RNA extraction

Grinding of all samples was performed using the MM200 instrument from Retsch. RNA extraction was performed according to the manufacturer's instructions using the E.Z.N.A™ Plant RNA Mini kit from Omega bio-tek. The exception was that 30 µl DEPC-treated water was used in the last step instead of 50-100 µl. RNA was quantified using Nano Drop ND-1000 (Fischer Science) and the quality was checked by analysis of the 260/280 ratio (1.9-2.0).

3.3 cDNA synthesis

One µg RNA was used for cDNA synthesis, the qScriptTM cDNA Synthesis Kit from Quanta biosciences was used according to the manufacturer's instructions. cDNA was quantified using Nano Drop ND-1000 (Fischer Science) and quality was checked with 260/280 ratio (1.6-1.8).

3.4 qPCR analysis

Throughout the experiments, the real-time PCR machine used was the 7000 Sequence Detection system from ABI PRISM. The Maxima SYBR Green/ROX qPCR Master Mix (2X) kit from Fermentas was used; the reaction mix contained 10 µl per reaction of PCR SYBR Green Master Mix, 1.2 µl (5µM) forward primer and reverse primer (Table 1) and 2.6 µl Milli Q water. 5 µl (50-100 ng/µl) cDNA was used for each reaction. The PCR program used was according to the manufacturer's instructions. The primers (Table 1) had already been designed according to

instructions from AB Applied Biosystems. An endogenous controls (*UBQ*) was used for relative gene expression and the ct values for the reference gene was constant for all samples. Throughout the experiments, three technical replicates were used.

Table 1. Primers used in the experiments performed.

Gene	Forward primer (5´to 3´)	Reverse primer (5´to 3´)
BAK1 (AT4G33430)	GGAATCAGAACTCTATCCTTGTGC	TTTGAGAGATCCAGAACTTGTAGC
BRI1 (AT4G39400)	AATTTCTCCGGTCCGATTCT	CTCCTGCAGAGTGTTTTTAGG
CPD1 (AT5G05690)	TGAAACAACCTCCACGATCA	TGCCCTAATCTTTTCATGCTCT
DET2 (AT2G38050)	GTAAAAACGGATTTCCGATCAC	TGTAATGCGAAACCCACCTC3
DWF4 (AT3G50660)	TTCTCGTTATGACCAACCTAATCTC	AGGATGACGCTCCGTTGTT
MPK4 (AT4G01370)	CAAGCAGACGCATCACAGTT	AAAATTGAACGGCCTCACAC
PR1a (At2g14610)	TGATCCTCGTGGGAATTATGT	TGCATGATCACATCATTACTTCAT
P.syringae (16S *rDNA*)	CAGCTCGTGTCGTGAGATGT	CACCGGCAGTCTCCTTAGAG
VSP2 (At5g24770)	GTTAGGGACCGGAGCATCAA	AACGGTCACTGAGTATGATGGGT

3.5 The expression of BR genes

3.5.1 A time course study of BR genes after priming with *B. amyloliquefaciens* 5113

A. thaliana Col-0 plants (control treated with water and another set primed with *B. amyloliquefaciens* 5113), grown under the same conditions as described above, were used in this experiment. The expression of BR-related genes (*DET2, BRI1, BAK1*) was analyzed at different time points (24 h, 3 days and 7 days) after *Bacillus* priming by soil drenching ($1x10^7$ CFU/mL). Two experiments were performed where gene expression was examined in either root or leaf at the different time points. RNA extraction, cDNA synthesis and qPCR were performed as described above.

3.6 To decipher the role of BR related genes upon priming and stress and their relation with JA/SA signaling

3.6.1 Basal level gene expression in Col-0, *bak1-4* and *det2*

Total RNA from leaves of wild type Col-0 and the two *Bacillus* primed ($1x10^7$ CFU/mL) BR mutant lines *bak1-4* and *det2* was extracted and cDNA synthesized. Real-time PCR analysis was performed with focus on gene expression of *PR1* (SA marker) and *VSP2* (JA marker).

3.6.2 BR gene expression of plants exposed to different treatments

RNA from water treated Col-0; *Bacillus* primed Col-0; *Bacillus* primed Col-0 challenged with *P. syringae* and Col-0 challenged with *P. syringae* was extracted. cDNA was synthesized and real-time PCR analysis was performed on BR biosynthetic genes (*DET2*, *DWF1*, *CPD1*), BR receptors (*BAK1*, *BRI1*) and SA signaling marker used as positive control for *P. syringae* infection (*PR1*).

The plants were inoculated with *P. syringae* DC3000 two days after priming with *B. amyloliquefaciens* 5113 ($1x10^7$/mL) spore suspension. *P. syringae* was grown overnight in Kings B medium and a suspension of $1*10^6$ CFU/mL in 10 mM $MgCl_2$ was used and syringe infiltrated on two leaves per plant.

3.7 To study if BR receptor is necessary for *Bacillus* colonization on plant roots

Seeds (Col-0, *bak1-4* and *det2*) were sterilized in 10 % chlorine and Tween 20 for 15 minutes and later in 70 % ethanol for 15 seconds followed by repeated (3x) washing in sterile water and then placed on MS media using TOP agar. The seeds were grown under the same conditions as described above. 40 ml of MS media (0.5xMS, 2 % sucrose and 1 % gelrite with a pH of 5.8) was poured on square plates (10x10cm). *A. thaliana* (Col-0, *bak1-4* and *det2*) seeds were placed on the MS plates in a row consisting of 6 plants/plate.

One set of the plants was primed with 5 µl of GFP tagged *B. amyloliquefaciens* 5113 ($1x10^7$ CFU/mL) to the root tip of the plants. An equal amount of sterile water was applied to the control plants. The colonization of plants was observed under microscope and the roots were harvested after 4 h, 6 h, 24 h and 4 days, and snap frozen in liquid nitrogen and stored in a -20°C freezer. Another set of plants (control and primed) were transferred to soil after 7 days in order to see if they were able to recover from the priming since *B. amyloliquefaciens* had colonized the plant more than expected.

One set of plants (Col-0, *bak1-4* and *det2*) were primed with 1 µl of *B. amyloliquefaciens* 5113 ($1x10^7$ CFU/mL) that had been heat killed at 99°C for 20 minutes in order to see if a surface molecule was responsible for priming. Sterile water and vegetative *B. amyloliquefaciens* 5113 were used as controls. The phenotypic character of the different plants was observed and the Col-0 (roots) harvested after 24 h. RNA was extracted from the root samples as described above. cDNA synthesis of Col-0 roots and real time PCR were as described above.

In order to be sure that the *Bacillus* spores actually had been killed by the high temperature, the bacteria were spread on MS plates and incubated at +28°C over night. This analysis indeed showed that the bacteria had not survived the heat treatment.

3.8 Phenotypic studies to elucidate the role of BRs upon priming and stress response

3.8.1 Priming

Three week old plants (*A. thaliana* Col-0 wild type and mutant line *bak1-4*) were soil-drenched ($1x10^7$ CFU/mL) with spore suspension of *B. amyliquefaciens* 5113. An equal amount of sterile water was applied to the control plants.

3.8.2 Herbivore and Pathogen

S. littoralis eggs were provided by Elisabeth Marling, SLU, Alnarp. The first instar larvae were applied to the leaves of the plants (one larva per plant for Col-0 and *bak1-4*) three days after priming. The larvae had only been exposed to artificial diet before applied to the leaves to avoid host plant adaptation. Larvae were collected 10 days after treatment, the mass was recorded and the phenotypic character of the plants was analyzed. Another set of plants (Col-0 and *bak1-4*) were challenged with *P. syringae* DC3000 grown on Kings media before use ($1x10^6$ CFU/mL in 10 mM $MgCl_2$) using a syringe on two leaves/plant three days after priming, the phenotypes of the plants were analyzed 10 days later.

Leaves challenged with *P. syringae* were harvested and pulverized by hand in a Falcon tube placed in liquid nitrogen. DNA extraction was performed with GeneMole from MoleGenetics

according to manufacturer's instructions. The DNA was concentrated using a SpeedVac concentrator from SAVANT and thereafter a qPCR was performed using 16S rDNA primers for *P. syringae* (Table 1).

4. Result

4.1 The expression of BR genes

4.1.1 A time course study of BR genes after priming with *B. amyloliquefaciens* strain 5113

In order to understand the role of BRs in plants upon *Bacillus* mediated priming, we carried out a time course study in *A. thaliana* wild type (Col-0) where root tips were treated with *Bacillus* spore suspension and samples were collected at different time intervals.

A six-fold up-regulation of *DET2* could be observed for the primed sample compared to the control in the leaf samples after 3 days while no difference was observed for the 24 h and 7 days samples (Figure 3A). As can be seen for the expression of *BRI1* in the leaf, a 3.5-fold up-regulation could be observed at 3 days. No difference was observed for the 24 h and 7 days samples (Figure 3B). Another gene examined was *BAK1*, where a 1.5 fold up-regulation in expression was observed for the 24 h sample compared to control, but no difference could be observed for the other time points (Figure 3C). *DET2* expression in the root samples of control- and primed samples did not differ in the 24 h sample or the 3 day sample. However it differed in the 7 day sample, the primed sample being 4-fold higher than the control (Figure 3D). The expression of *BRI1* in the root of control and primed plants showed the same pattern; the 24 h and the 3 day samples did not differ, but the primed sample for 7 days was increased 2-fold (Figure 3E). The expression of *BAK1* in the root sample of the control and the primed sample did not differ at 24 h, but were 2-fold higher at 3 days and 7 days for the primed samples (Figure 3F).

Leaf Root

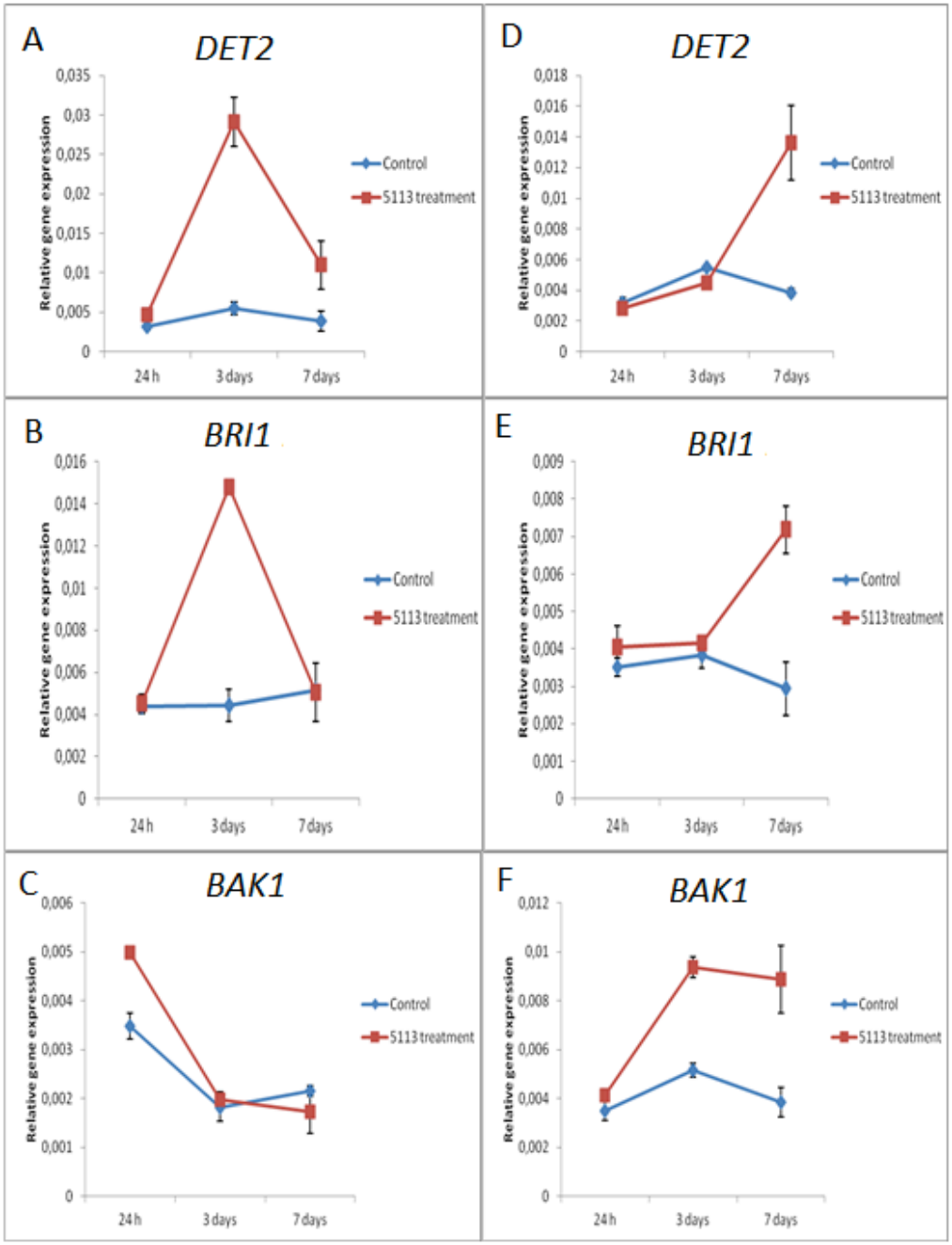

Figure 3. Expression of the BR-related genes *DET2, BRI1* and *BAK1* in leaves of wild type Col-0 compared to roots over time (24h, 3 days and 7 days) after priming. Six plants were pooled together as one replicate for RNA extraction and used for qPCR analysis. The experiment was repeated twice.

4.2 To decipher the role of BR related genes upon priming and stress and their relation with JA/SA signaling

4.2.1 Basal level gene expression in Col-0, *bak1-4* and *det2*

In order to see if a lower amount of endogenous BRs or a lack of BR co-receptor is having any impact on JA or SA signaling, qPCR analysis of JA marker gene *VSP2* and SA marker gene *PR1* was carried out on the BR mutant lines *det2* and *bak1-4*.

The basal expression of *PR1* in the *bak1-4* mutant was 6-fold higher compared to Col-0 and the expression of *det2* was down-regulated to one third (Figure 4). Considering the expression of *VSP2*, a down-regulation to one eight could be observed in the *bak1-4* mutant compared to Col-0. The same result was obtained for the *det2* mutant (Figure 5).

These results indicated that there could be much lower levels of JA or SA in the *det2* mutant and also that there is more SA than JA in the *bak1-4* mutant.

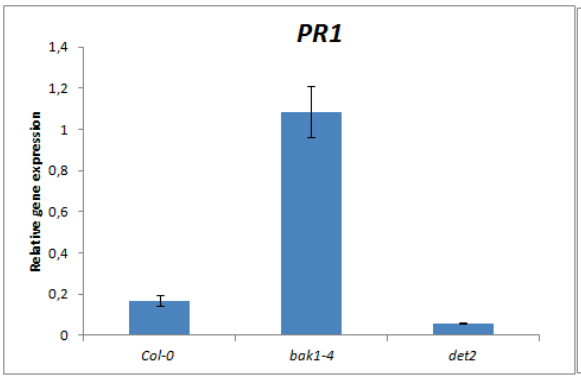

Figure 4. qPCR analysis on *Col-0*, *bak1-4* and *det2* with primers for *PR1*. Six plants were pooled together as one replicate for RNA extraction. The experiment was performed once.

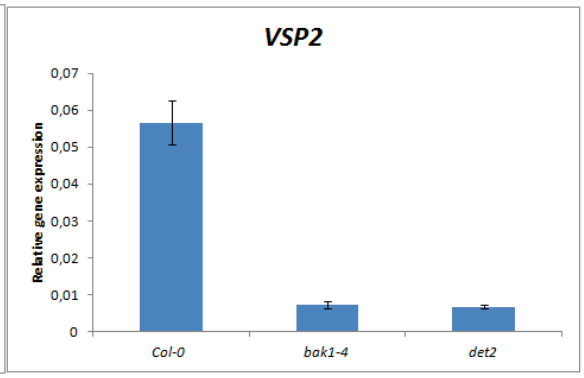

Figure 5. qPCR analysis on *Col-0*, *bak1-4* and *det2* with primers for *VSP2*. Six plants were pooled together as one replicate for RNA extraction The experiment was performed once.

4.2.2 BR gene expression of plants exposed to different treatments

To further study the role of BRs in disease resistance, BR related gene expression was studied in order to see how the genes were regulated upon priming and when exposed to the hemi-biotrophic pathogen *P. syringae*.

The expression of BR biosynthetic genes (*DET2*, *DWF1*, *CPD1*), BR receptors (*BAK1*, *BRI1*) and *PR1* (SA signaling marker used as positive control for *P. syringae* infection) was studied in *A. thaliana* wild type (Col-0) primed and non-primed with *B. amyliquefaciens* and subsequently challenged with *P. syringae*. Water treated plants were used as controls.

qPCR analysis was performed on the leaves of the challenge inoculated and mock-inoculated plants. *BRI1* gene expression was found to be 3-fold up-regulated in the leaves of *Bacillus* primed and *P. syringae* challenged plants compared to the mock inoculated plants. A similar level of expression was observed in the plants primed with *Bacillus* but not challenged with the pathogen (Figure 6A). Considering the expression of *BAK1*, the primed and pathogen challenged plants

showed a 3-fold up-regulation compared to control. Non-primed plants exposed to *P. syringae* showed a 4-fold up-regulation compared to the mock-inoculated plants (Figure 6B). The expression of *CPD* in the *Bacillus* primed samples showed a slight up-regulation compared to the non-primed. Primed and non-primed plants exposed to pathogen showed to be reduced by half compared to the control (Figure 6C). The expression of *DET2* in the primed sample was up-regulated 3-fold and 8-fold in the primed sample exposed to pathogen and a 7-fold up-regulation was observed for the sample only exposed to pathogen compared to the control (Figure 6D). *PR1* was used as a positive control for SA and was reduced to one fifth for the primed sample, but a 11-fold up-regulation in the primed sample exposed to pathogen and a 10-fold up-regulation in the sample only exposed to pathogen (Figure 6E). Whereas, a down regulation by one third of *DWF1* was observed in both primed samples compared to control and plants only exposed to *P. syringae* (Figure 6F).

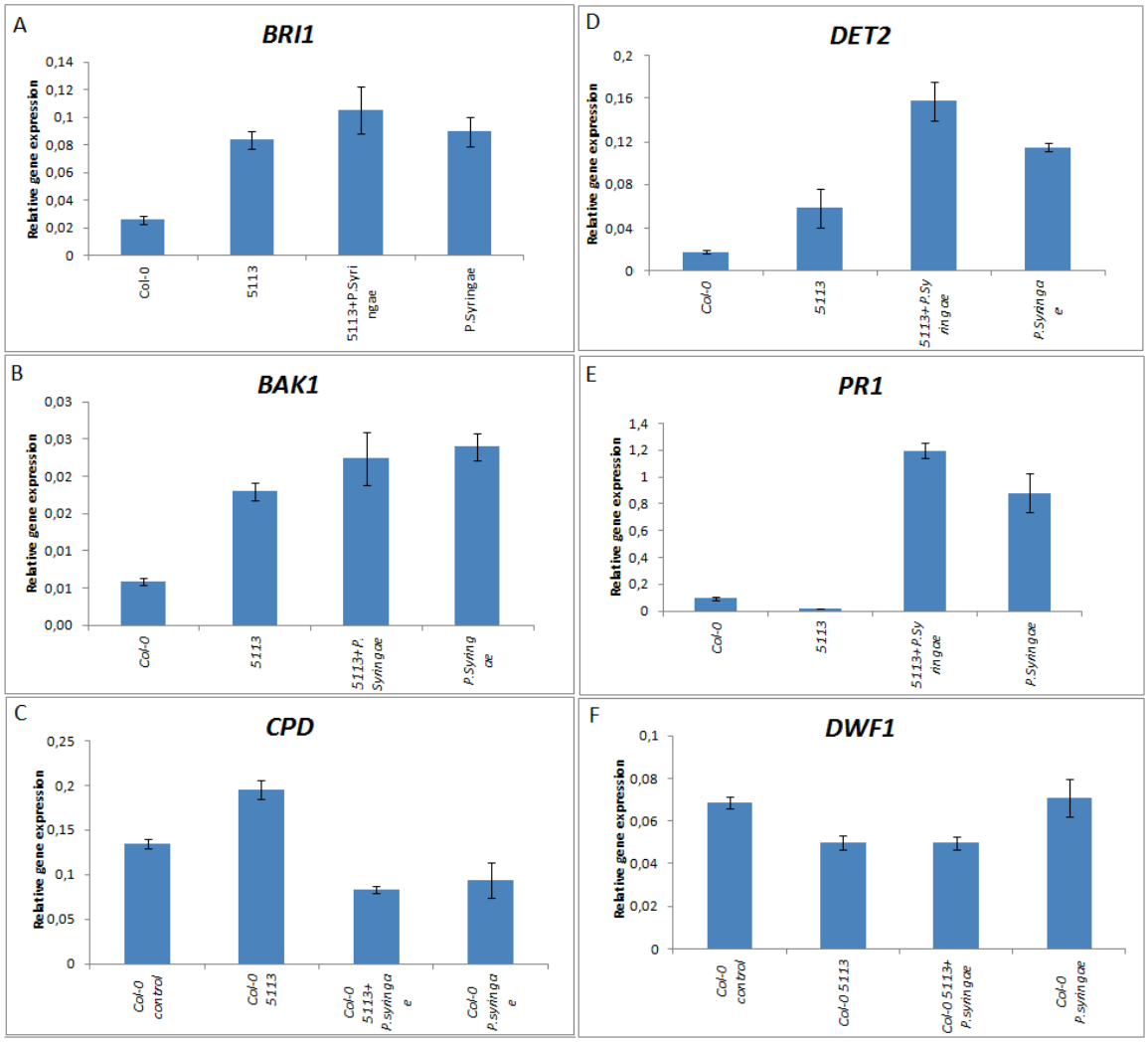

Figure 6. qPCR analysis of cDNA from untreated Col-0, primed Col-0, primed Col-0 exposed to *P. syringae* and Col-0 exposed to *P. syringae*. Gene expression for (A) *BRI1*, (B) *BAK1*, (C) *CPD*, (D) *DET2*, (E) *PR1* and (F) *DWF1* was examined. Six plants were pooled together as one replicate for RNA extraction and used for qPCR analysis. The experiment was performed once.

4.3 To study if BR receptor is necessary for *Bacillus* colonization on plant roots

A GFP tagged strain of *B. amyloliquefaciens* 5113 was used to study the colonization pattern in the wild type Col-0 and mutant lines *det2* and *bak1-4*. Considering the result above, it was of interest to study further if endogenous BR levels or the BR receptor played any role for colonization to occur in the roots. A time course study was performed on *A. thaliana* Col-0 plants after root dip treatment with GFP tagged *B. amyloliquefaciens*.

24 h after *Bacillus* treatment: When a visual comparison was performed between the phenotypes for Col-0 control and Col-0 primed plants, they differed in such a way that the primed plants had more lateral roots and slightly larger leaves than the control (Figure 7a/b). Primed *bak1-4* had slightly more lateral roots than the control but the difference was not significant (Figure 7c/d). The *det2* mutant had most lateral roots of all plants, but the smallest leaves. A phenotypic difference observed was that the primed plants had more lateral roots than the control also for *det2* (Figure 7e/f).

Four days after *Bacillus* treatment: The plants that were harvested after four days had a general pattern where primed plants had much lateral roots compared to the control plants except for *bak1-4* mutants (Figure 8). As can be seen in Figure 8 and 9, the colonization of *Bacillus* in Col-0 was very successful and more lateral roots could be observed for the primed sample than the control. The *bak1-4* mutant showed a phenotype with shorter main and lateral roots than both Col-0 and *det2*, but generally more bacteria colonizing the plants (Figure 8 and 10). The *det2* mutant had most lateral roots of all plants, as in the 24 h plants and more lateral roots could be observed for the primed plants (Figure 8 and 11). Colonization of *Bacillus* was successful in the *det2* mutant as well (Figure 11b).

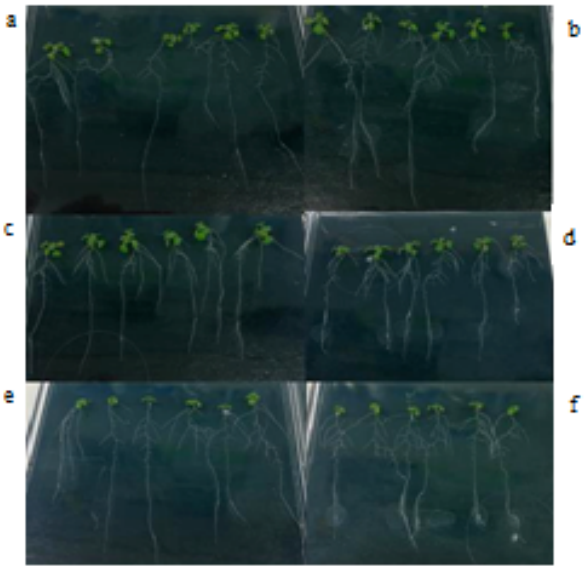

Figure 7. *Bacillus* colonization of *A. thaliana* plants. Comparison between primed (*B. amyliquefaciens* 5113) and unprimed plants of *A. thaliana* 24 h after priming. (a) Col-0 control, (b) Col-0 primed (c) *bak1-4* control d) *bak1*-4 primed (e) *det2* control (f) *det2* primed.

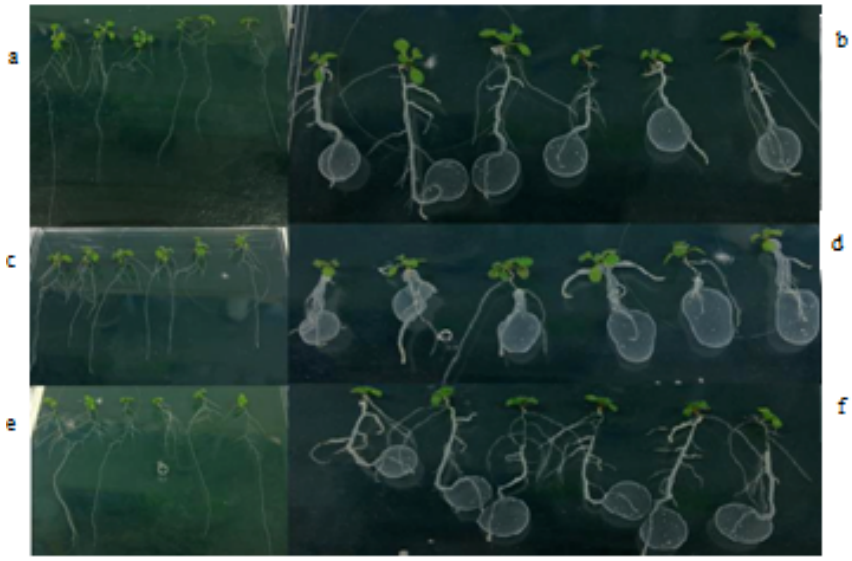

Figure 8. *Bacillus* colonization of *A. thaliana* plants. Comparison between primed (*B. amyliquefaciens* 5113) and unprimed plants of *A. thaliana* 4 days after priming. (a) Col-0 control (b) Col-0 primed (c) *bak1-4* control (d) *bak1-4* primed (e) *det2* control (f) *det2* primed.

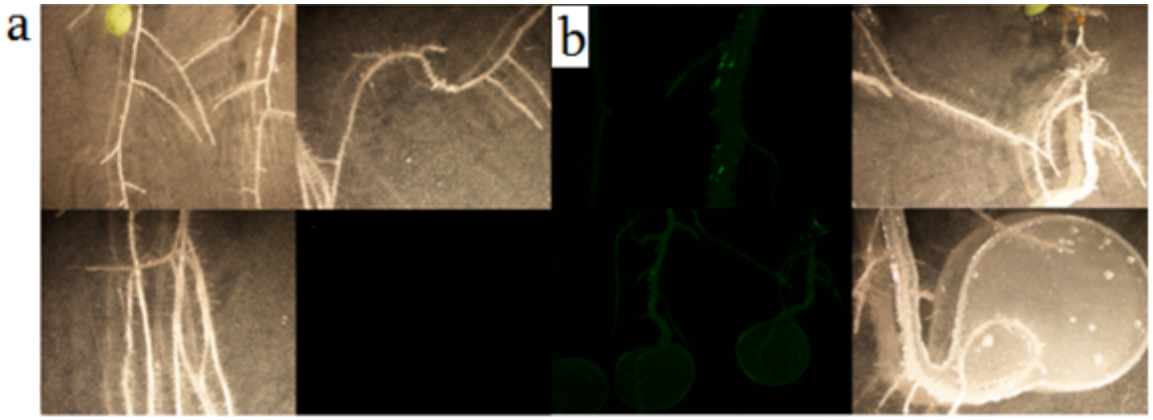

Figure 9. *Bacillus* colonization of *A. thaliana* plants. (a) Col-0 control (b) primed Col-0 after 4 days. The primed plant was colonized by GFP tagged *B. amyliquefaciens* 5113.

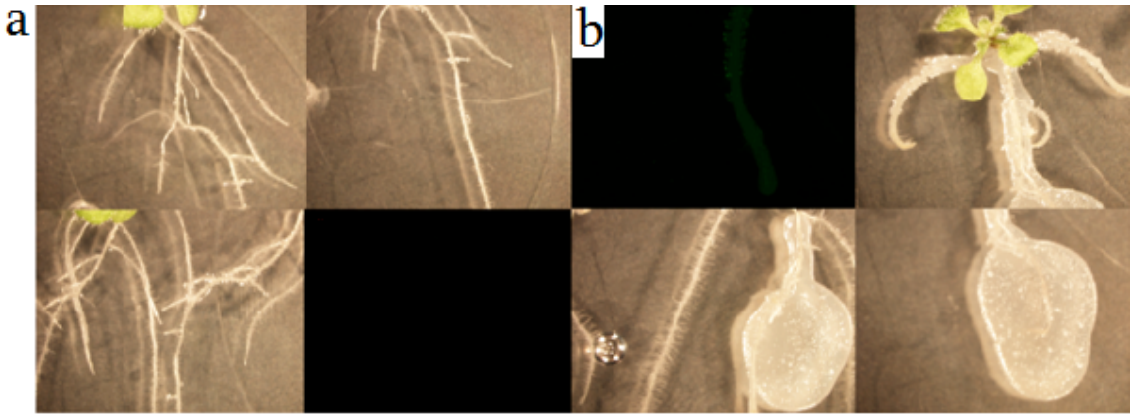

Figure 10. *Bacillus* colonization of *A. thaliana* plants. (a) *bak1-4* control (b) primed *bak1-4* after 4 days. The primed plant was colonized by GFP-tagged *B. amyliquefaciens* 5113.

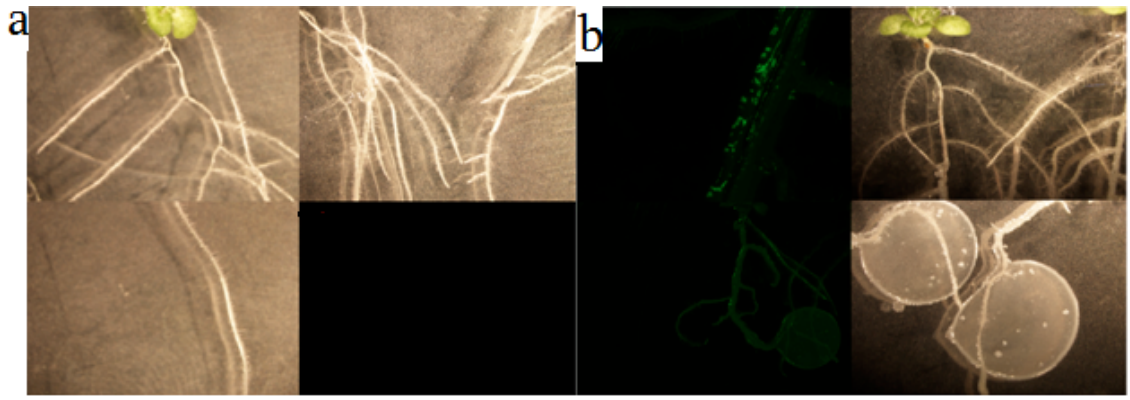

Figure 11. *Bacillus* colonization of *A. thaliana* plants. (a) *det2* control (b) primed *det*2 after 4 days. The primed plant is colonized by *B. amyliquefaciens* strain 5113 with GFP attached.

Seven days after *Bacillus* treatment**:** Plants of primed wild type Col-0 can be seen below in Figure 12. A comparison can be made to mutant *bak1-4* in Figure 13, where the roots were much shorter and the colonization comprehensive. The plant that was least colonized was the BR mutant *det2*, which also was the plant with the smallest leaves. The *det2* mutant was also the plant with the longest roots when comparing the primed plants (Figure 14). The observations performed were visual and not measured.

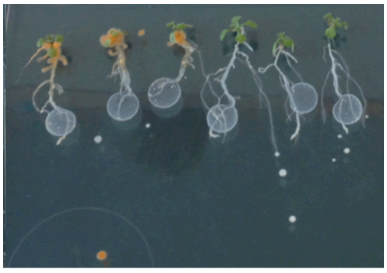

Figure 12. Primed Col-0 after 7 days.

Figure 13. Primed *bak1-4* after 7 days.

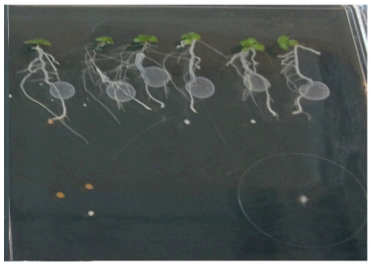

Figure 14. Primed *det2* after 7 days.

The recovery of the plants transplanted to soil showed a dramatic visual difference between the control and the primed plants, the primed plants being superior (Figure 15). The difference between the control and the primed plants was dramatic, the primed plants were much larger and a greater number of plants survived the transplantation. The *det2* mutant was the plant that recovered worst, both for primed and control, the recovery was however slightly better for the primed plants. There was also a difference in flowering time, the primed plants being faster (15b). All comparisons performed were visual and not measured.

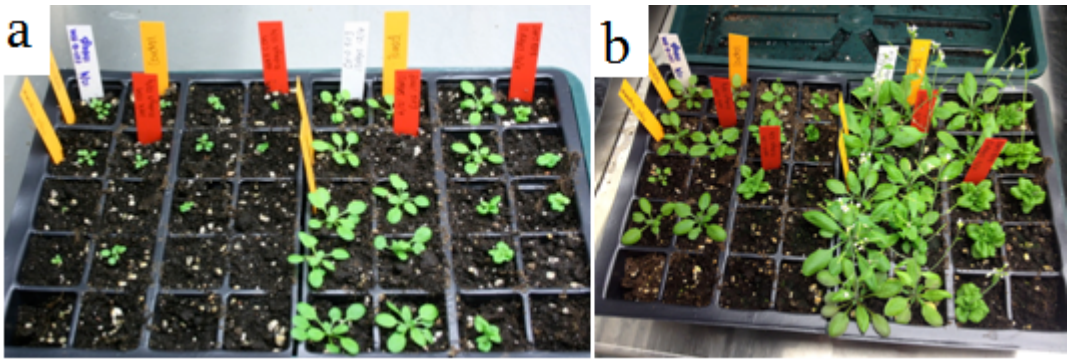

Figure 15. Recovery of control plants (left) versus primed plants (right) of Col-0, *det2* and *bak1-4*. The first picture (a) was taken after ten days and the second (b) after three weeks.

In order to understand the role of BR receptors upon *Bacillus* contact on plants roots, a time course study (3h, 6h, 24h and 4 days) to study the gene expression of BR receptors BRI1 and BAK1 was carried out on *A. thaliana* wild type Col-0. *BAK1* expression was observed to be reduced by half in the primed samples at 3h, 6 h and 24 h compared to the non primed controls, whereas after 4 days, the expression levels of primed samples returned to the basal level of the control samples (Figure 16).

The expression of *BRI1* did not differ between the primed and the control samples at any time point (Figure 17).

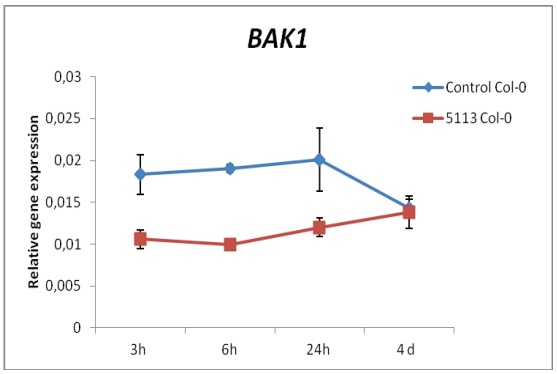

Figure 16. *BAK1* expression in roots of *A. thaliana* wild type Col-0 over time (3h, 6h, 24h and 4 days). Six plants were pooled together as one replicate for RNA extraction and used for qPCR analysis. The experiment was repeated twice.

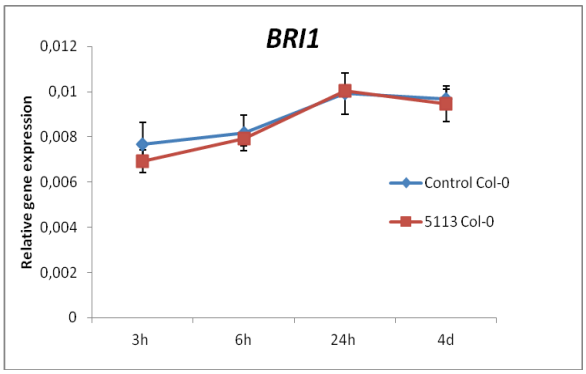

Figure 17. *BRI1* expression in roots of *A. thaliana* wild type Col-0 over time (3h, 6h, 24h and 4 days). Six plants were pooled together as one replicate for RNA extraction and used for qPCR analysis. The experiment was repeated twice.

Gene expression of *MPK4* was studied in Col-0 and *bak1-4* to see if *BAK1* has an impact on the MAMP response in plants upon contact with *Bacillus* (Figure 18 and 19). The MAMP response was observed rather early in Col-0. Already after 6 h an almost 2-fold up-regulation could be observed for the primed sample, which suggests MAMP recognition of *Bacillus*. While at 4 days a down regulation by a fourth was observed in the primed roots compared to the non-primed (Figure 18). Considering the mutant *bak1-4*, the gene expression was reduced by half for the 6 h primed sample. The 3 h, 24 h and 4 days samples did not differ between the control and primed samples (Figure 19).

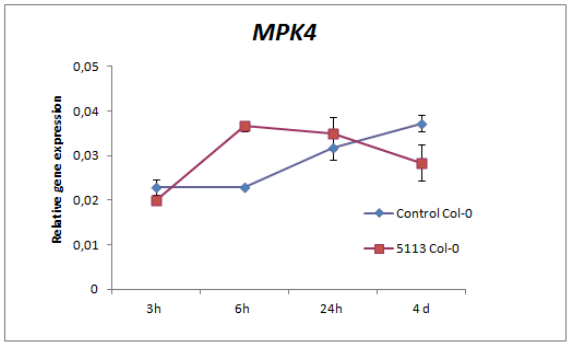

Figure 18. Expression of *MPK4* in roots of *A. thaliana* wild type Col-0. Six plants were pooled together as one replicate for RNA extraction and used for qPCR analysis. The experiment was repeated twice.

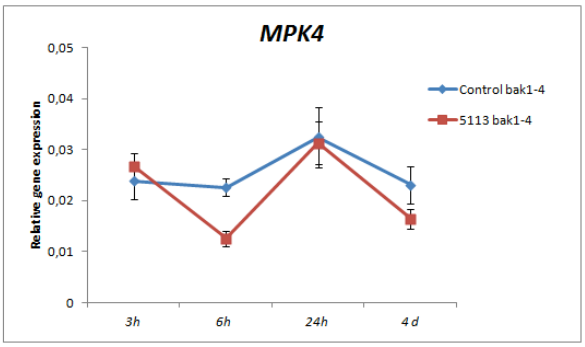

Figure 19. Expression of *MPK4* of roots in *A. thaliana* mutant *bak1-4*. Six plants were pooled together as one replicate for RNA extraction and used for qPCR analysis. The experiment was repeated twice.

The phenotypes of the plants primed with heat killed *B. amyloliquefaciens* did not differ greatly from the plants primed by vegetative *Bacillus*. When a visual comparison was made after one week, the Col-0 plants root primed with heat killed *B. amyloliquefaciens* had larger leaves than the water control, but did not differ from the plants primed by vegetative *Bacillus*. The roots of both types of primed plants had also more lateral roots than the water control. Vegetative *Bacillus* primed *bak1-4* had much more lateral roots than the water control and slightly more roots than the plants primed with heat killed *Bacillus*. Considering the *det2* plants, the side roots of both types

of primed plants were more similar to each other than to the water control, being shorter but in a greater amount. This was consistent with the phenotype observed in the previous experiment.

The relative gene expression of *BAK1* was analyzed for wild-type Col-0, water control, Col-0 primed with vegetative *Bacillus* and Col-0 primed with heat-killed *Bacillus* (Figure 20). The gene expression of *BAK1* was highest in the unprimed plants and rather similar for the two types of primed plants.

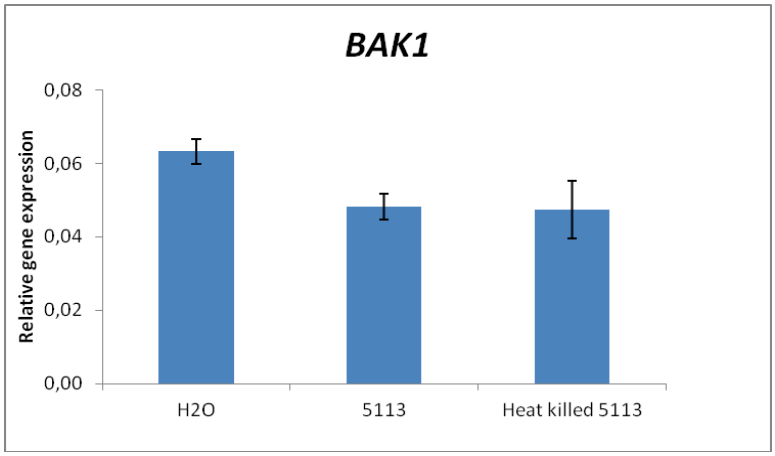

Figure 20. Gene expression of *BAK1* in roots of *A. thaliana* Col-0 control, Col-0 24 h after primed with *B. amyloliquefaciens* 5113 and Col-0 primed with heat killed *B. amyloliquefaciens* 5113. Six plants were pooled together as one replicate for RNA extraction and used for qPCR analysis. The experiment was performed once.

4.4 Phenotypic studies to elucidate the role of BRs upon priming and stress response

The effect of priming was observed in plants exposed to herbivore and pathogen attack. It was of interest to study the expression of the gene *BAK1* involved in MAMP signaling.

Herbivore bioassay:
A generalist herbivore bioassay with the larvae of *S. littoralis* was carried out on three-week-old Col-0 and *bak1-4* plants. The larval weight in the different groups obtained was divided into groups of similarity (Figure 21) according to an ANOVA test and it was shown that there were no significant differences between the larvae. As can be seen in Figure 21, the larvae that had fed on the mutant *bak1-4* were slightly larger than larvae that had fed on Col-0. Two outliers with a greater mass than the other larva were observed in the *Bacillus* treated Col-0. There was also a difference between the controls and the primed samples where the larvae that had fed on the primed samples had a lower weight, also demonstrated in Figure 21, this is however not significant. Phenotypic analysis of the larvae (Figure 22) showed that the larvae from the *bak1-4* mutants were slightly larger considering the mass than the other larvae. What also is of interest is the fact that there were fewer larvae remaining in the Col-0 and *bak1-4* primed samples than in the controls (Table 2). Out of 10 larvae 8 had survived on wild type Col-0 control and 10 out of 10 larvae on the mutant *bak1-4* control in comparison to 5 out of 10 for wild type Col-0 primed and 7 out of 10 on the primed mutant *bak1-4*.

The larvae seemed to have eaten a lot on the Col-0 control (Figure 23a) and not at all as much on the primed Col-0 plants (Figure 23b). Considering the mutant *bak1-4*, no major difference in the phenotype could be seen for the control and the primed plants (Figure 23c/d).

Table 2. Average weight of larvae taken from *A. thaliana* wild type Col-0 and the mutant line *bak1-4.* The number of remaining larvae from each plant line (out of ten from the beginning of the experiment) is indicated.

Control plants	**Mean weight (g)**	**Number of larvae**	**Primed plants**	**Mean weight (g)**	**Number of larvae**
Col-0	0.0213	8/10	Col-0	0.0223	5/10
bak1-4 mutant	0.0195	10/10	*bak1-4* mutant	0.0315	7/10

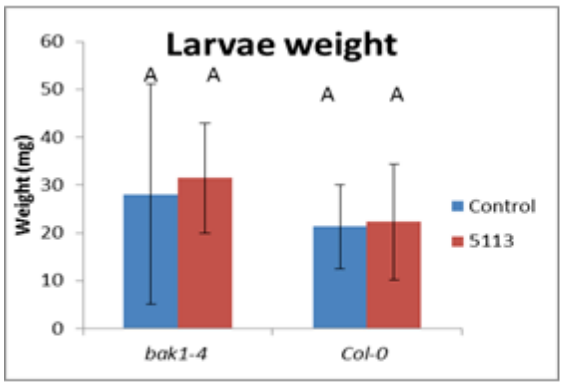

Figure 21. Larvae weight after feeding on Col-0 and *bak1-4*. Letters indicates results of an ANOVA test.

Figure 22. Larvae from A) Col-0 control; B) *bak1-4* control; C) Col-0 primed; D) *bak1-4* primed.

Figure 23. Appearance of *A. thaliana* plants after treatment with *S. littoralis*, control and primed plants. (A) Col-0 control, (B) Col-0 primed, (C) *bak1-4* control, (D) *bak1-4* primed.

Pathogen response:
Three-week-old *A. thaliana* Col-0 and *bak1-4* plants were challenge inoculated with pathogen *P. syringae* DC3000. The result from the *P. syringae* post inoculation had a similar trend to the bioassay with *S. littoralis*. The chlorotic symptoms observed upon *P. syringae* inoculation were more in the non-primed plants but the symptoms in the primed plants were restricted to cell death (Figure 25 a/b). A higher degree of chlorosis was observed in the *bak1-4* plants compared to the Col-0 (Figure 25 c/d) but there were no phenotypic difference between the primed and non-primed *bak1-4* plants. In Col-0, the symptoms could be seen only on the infected leaves, which was not the case considering the control for *bak1-4* where they had spread. qPCR analysis of *A. thaliana* infected with *P. syringae* showed a higher concentration of the pathogen *P. syringae* in the non-primed plants (Figure 24), compared to the similar result in the phenotypic observation. When Col-0 was compared to *bak1-4*, there was a higher level of *P. syringae* in the mutant *bak1-4*.

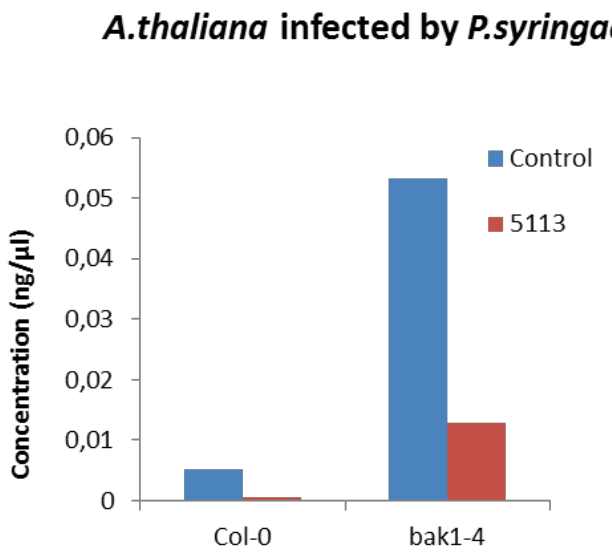

Figure 24. qPCR quantification of *P. syringae* in control- and primed samples. The inoculated leaves from five plants were were pooled together and used for qPCR analysis. This experiment was carried out only once.

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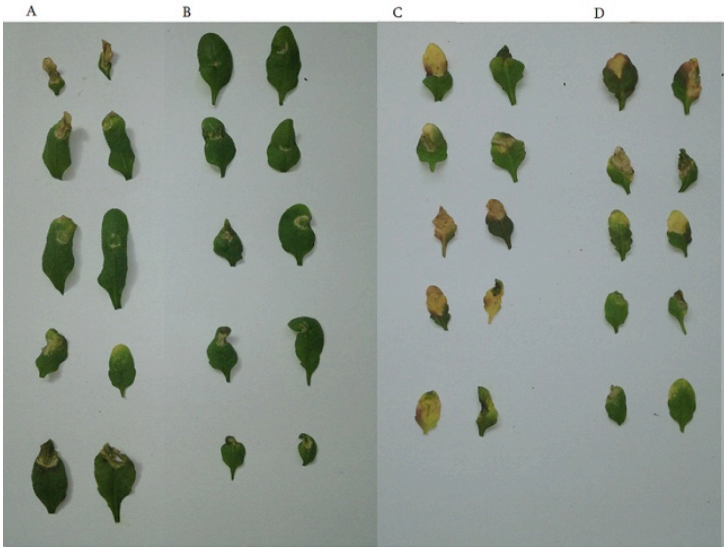

Figure 25. Symptoms on *A. thaliana* after challenge inoculation with *P. syringae*, for control and primed plants. A) Col-0 control, B) Col-0 *Bacillus* primed, C) *bak1-4* control, D) *bak1-4 Bacillus* primed.

5. Discussion

5.1 The expression of BR genes

5.1.1 A time course study of BR genes after priming with *B. amyloliquefaciens* 5113

Priming by *Bacillus* to the roots of *A. thaliana* wild type Col-0 had a great impact on BR related genes both locally (roots) and systemically (leaves). The BR-related genes *DET2, BAK1* and *BRI1* were expressed differentially in the leaves and roots of Col-0 over different time points. The response for all genes seems to be faster in leaves than in roots, which could be an evolutionary advantage since leaves are less protected than roots. Considering the expression of *BAK1* in the leaves, a 1.5 fold up-regulation was observed at 24 h in the primed sample compared to the non-primed. The response of *BAK1* in the root samples was not as fast as that of the leaves. The differential expression pattern observed in the non-primed samples also suggests a developmental role of *BAK1*, which has also been previously reported (Kemmerling and Nürnberger, 2008). In addition the up-regulated expression of *DET2* upon priming might indicate an increased accumulation of endogenous levels of BRs which supports the phenotypic data observed previously for the primed plants being larger in size compared to the non-primed.

5.2 The role of BR related genes upon priming and stress and their relation with JA/SA signaling

5.2.1 Basal level gene expression in Col-0, *bak1-4* and *det2*

VSP2 and *PR1* are two genes involved in different defense responses. While *VSP2* is a JA-responsive marker gene, *PR1* is a SA-responsive marker gene (Lebel *et al*. 1998; Liu *et al*. 2006). The result from the qPCR analysis showed that the expression of *PR1* (and thereby also the SA level) in the mutant *bak1-4* was high. Since the SA and JA pathways are antagonistic, the low level of JA in the *bak1-4* mutant will allow a high level of SA (Pieterse *et al*. 2009). It is a possibility that BRI1 can associate with an unknown protein that negatively can regulate SA-dependent responses and that *BAK1* indirectly can affect SA-dependent pathways. Considering *VSP2* it could be the case that low levels of BR negatively regulates JA dependent pathways or that BR is needed for downstream signaling. It would have been interesting to compare the levels of BR in wild type Col-0, mutant *det2* and *bak1-4* upon subsequent challenge inoculations with pathogens in order to see if BR could have an influence.

5.2.2 BR gene expression of plants exposed to different biotic stress conditions

To understand what genes are involved in priming, one can use mutants and observe the expression of the genes upon priming. In this experiment, there was an up-regulation of *BAK1* transcripts in the wild type samples primed with *Bacillus* and/or challenged with *P. syringae*. This result is very similar to the *BRI1* expression. These data suggests that *BAK1* and *BRI1* are involved in MAMP/PAMP triggered responses. Since BRI1 is the BR receptor and BAK1 is the co-receptor, there is probably an interaction between them during priming for BR signaling to take place. *DET2* is, as mentioned before, involved in biosynthesis of BRs and is up-regulated upon priming and also in defense. *DET2* could be rate-limiting for BR production and more important than the down stream steps involving *CPD* and *DWF1*. The expression of *CPD* in the primed sample was slightly up-regulated, but it is difficult to say if *CPD* is affected by priming. It is down regulated upon pathogen attack though. *PR1* (pathogenesis-related protein 1) is normally induced during SAR and, as can be seen in Figure 6, *PR1* is not up-regulated until exposure to *P.*

syringae. *Bacillus* treatment alone did not significantly alter the *PR1* expression levels, but did potentiate the up-regulation in response to pathogen inoculation. The down-regulation of *PR1* upon priming could indicate a down-regulation in pathogen triggered immunity. The *DWF1* gene was slightly down-regulated upon priming and further research is needed to investigate this phenomenon, but it seems to be affected by priming. In short, it seems that the BR-associated genes *BAK1, DET2, BRI1* and *DWF1* could be involved in the plant´s response to priming by *B. amyloliquefaciens* 5113, however more research is needed in order to confirm this.

5.3 Is the BR receptor necessary for *Bacillus* colonization on plant roots?

In order to elucidate the role of BR and BR receptors on the colonization pattern of *B. amyloliquefaciens*, studies were carried out in different BR mutants and the wild type. As mentioned before, plants are able to recognize conserved epitopes, MAMPs and they are involved in ISR (Millet *et al*. 2010). It is of interest to examine whether the BAK1 receptor alone can be activated by *B. amyloliquefaciens* and if this results in a downstream process in the plant leading to a defense response (Figure 26). What also is of interest is whether it is necessary for the bacteria to be alive for priming to take place. If a surface molecule or some metabolite is responsible, maybe priming can take place even though the bacteria are dead?

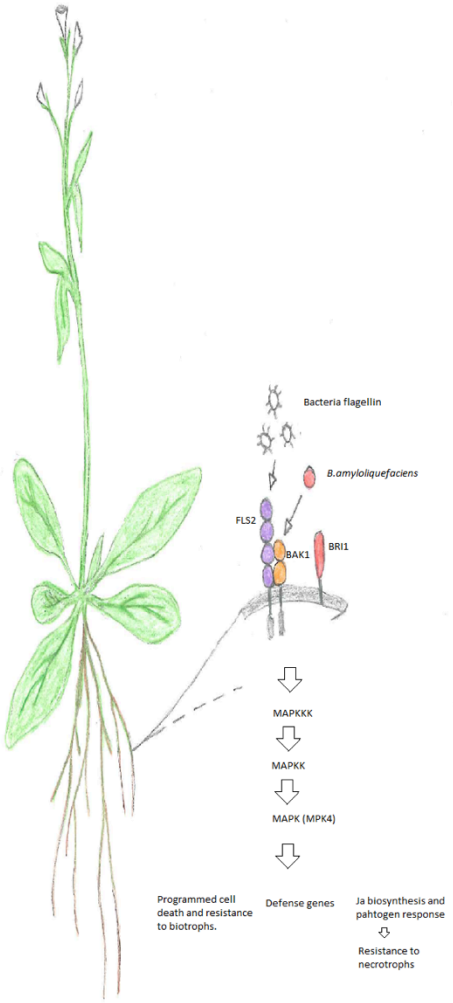

Figure 26. Activation of the BAK1 receptor by *Bacillus*, leading to a cellular defense response. (Modified from Panstruga *et al*. 2009)

More lateral roots for the primed plants were generally observed (24h and the 4 days samples), something that can be very beneficial for the plant since it can increase the ability to take up and store nutrients; this indicates that the beneficial bacteria are able to induce growth of side roots. An increase in the root biomass of primed plants has been shown in earlier research in oilseed rape (Bejai *et al*. 2009). Another observation for the 24 h Col-0 was that the leaves were larger for the primed plant than for the control, which could mean that priming by *Bacillus* triggers some BR-related genes. The fact that this phenomenon could not be seen in the two mutants is interesting. *DET2* is involved in BR biosynthesis and there is a lack of BR in the *det2* mutant, which could be one explanation for the results. BAK1 is involved in BR signaling, being the co-receptor of BRI1. Accordingly, when BAK1 is absent it is possible that the signaling is not fully functional and that the biosynthesis of BR is reduced (Eckardt 2005).

The colonization of plants after 4 days was very extensive and it might have been better to use a smaller amount of bacteria for the priming. The *det2* mutant was the least colonized plant after

seven days, which could mean that BR is necessary for colonization since the mutant only contains less than 10 % of wildtype BR levels (Noguchi *et al*. 1999). What could be done to test this hypothesis is to apply 24 epi-brassinolide (Brassinolide), which is one of the most effective BRs and can restore the mutant to a normal phenotype (Fujioka *et al*. 1997; Nakashita *et al*. 2003). *Bacillus* would later be applied to see if normal colonization would take place. Future research could also be to observe differences in MAMP response in the *det2* mutant compared to wild type Col-0. The result also showed a difference in the colonization of *bak1-4* compared to Col-0 and *det2, bak1-4* being more colonized. This could mean that *BAK1* might be playing a role during *Bacillus* colonization in *A. thaliana*. It would have been interesting to perform more research on a *bak1-4* mutant to see how functional the ISR is in this mutant. One could for example look in to the efficiency of priming upon inoculation with other necrotrophic pathogens in *bak1-4*. What also could have been done would be to use mutant lines of *bri1* that lack the BR receptor and see if colonization still could take place.

The recovery of the plants on soil after colonization by *Bacillus* showed a surprising difference between the primed plants and the controls, where the primed plants were superior. This indicates that *Bacillus* is able to help the plant in the recovery and handle the stress it is exposed to when the environment is changed. It was interesting that the primed *bak1-4* plants also recovered better than the control since a lot of the results indicate that *BAK1* is important for priming to take place. This can be explained by the fact that the receptor in BR signaling for BR is BRI1 and BAK1 is only the co-receptor and probably not necessary for full activation (Eckardt 2005). The mutant *det2,* however, does not have a functional BR biosynthesis and was therefore not expected to handle the recovery very well. It is, however, possible that the *Bacillus* is able to induce a BR like hormone that helps *det2* in the recovery.

The BR associated gene *BAK1* was down regulated in primed roots for the 3 h, 6 h and 24 h samples, which was unexpected since the beneficial bacteria are supposed to protect the plant and not lower its defense. However, the expression levels of primed samples returned to basal levels for the 4 days sample. This result most likely means that the MAMP signaling and thereby ISR was prevented in order for colonization by the *Bacillus* to take place. When colonization has been completed, the *Bacillus* can start helping the plant by inducing a latent defense. This phenomenon has been shown for other beneficial bacteria like *P. fluorescense* WCS417r (Millet *et al*. 2010). Another explanation, as mentioned by Millet *et al*. (2010), is that the beneficial bacteria need to be protected against MAMP-induced antimicrobial secretions, if MAMP is suppressed the bacteria will be protected. The expression of *BRI1* during the same time course in the primed sample did not differ from the control. This means that there is no down- or up-regulation of *BRI1* during colonization of *Bacillus*. There could, however, be a difference at protein level, which is not observed at transcription level.

The up-regulation of the gene *MPK4* involved in the MAMP recognition indicates that there is a rather fast defense response to priming. The up-regulation could be observed in Col-0 after only 6 h. BAK1 could be the receptor activated since *MPK4* is down regulated to basal levels at 6 h in the mutant *bak1-4*. What happens on protein level has not been investigated and there could be a difference between protein and transcript levels.

When comparing the plants primed with vegetative *Bacillus*, the heat killed *Bacillus* and the water control, the differences between the primed plants were minor and the water control differed more from the primed samples for Col-0 and *det2*. The mutant *bak1-4* primed with heat killed *Bacillus* had more similarities to the water control and it is possible that the bacteria could not colonize the *bak1-4* mutant very efficiently. From the qPCR data for wild type Col-0, one can see that the *BAK1* expression is the highest in the unprimed sample and that it is down-regulated

slightly upon priming, no matter if the bacteria were heat killed or vegetative, which is consistent with earlier data for the regular 24 h priming. This indicates that there might be a surface molecule on the bacteria that is involved in priming and binding to *BAK1*. The gene expression of *BAK1* in Col-0 control is 3-fold (Figure 20) compared to the expression of *BAK1* in Figure 16 and the expression of *BAK1* in the primed sample is 5-fold (Figure 20) compared to Figure 16. This could be explained by the fact that the plant material used in Figure 20 was older when the plants were primed and more material could be collected at 24 h.

5.4 Phenotypic studies to elucidate the role of BRs upon priming and stress response

When *A. thaliana* plants were challenged with *S. littoralis*, a great difference could be observed between the control and primed plants for Col-0. The primed plants had managed the stress better than the control plants, indicating that priming using *B. amyloliquefaciens* 5113 is efficient on *A. thaliana* as protection against herbivore. Some of the larvae from the primed plants (Col-0 and *bak1-4*) could not be collected, which indicates that the primed plants were protected to some extent from the larvae and the larvae then preferred eating other larvae. When *BAK1* is mutated, this will lead to lower levels of JA (*BAK1* can probably post-transcriptionally affect the activity of some biosynthetic JA enzymes), which is interesting since JA is involved in herbivore defense. This will occur in plants that are wounded, as in this case when under herbivore attack, which will promote the larvae to eat more (Yang *et al*. 2011). Since the larvae from the primed *bak1-4* mutants were rather large, it is possible that this was the case. The larvae were divided into groups of similarity and there were no significant difference between the larvae from the different plants. It should be noted though, that the *Bacillus* treated Col-0 contained two outliers of larvae that had a significantly greater mass than the remaining larvae. In order to trust the grouping completely, the experiment needs to be repeated.

The *bak1-4* mutant of *A. thaliana,* which does not have the co-receptor for BR synthesis, showed no phenotypic difference between the control and the primed plants when the larvae were removed, nor when *P. syringae* had been used, which once again indicates that *BAK1* is of great importance for priming to take place. The leaves infected with *P. syringae* from the qPCR analysis showed a difference in the amount of pathogen between Col-0 and *bak1-4*, with *bak1-4* having a greater amount of *P. syringae*. There was a large difference between the control and the primed sample of *bak1-4* considering the concentration of *P. syringae* (Figure 24), the primed sample having a smaller concentration of the pathogen. This could mean that *BAK1* is very important in plant defense response against *P. syringae*, but also that priming has a small effect on the plant even though *BAK1* is not present.

6. Conclusion

The experiments performed showed a dramatical difference between the control and the primed plants where one could come to the conclusion that the primed plants were better in handling stress of different kinds. Upon *Bacillus* treatment, the BR biosynthetic gene *DET2* was up-regulated in leaves and roots. *BAK1* seemed to play a prominent role in the defense response in *A. thaliana*. This study has revealed the suppression of MAMP responses by *Bacillus* as reflected by down-regulation of *BAK1* for successful colonization and subsequent activation of ISR. A surface molecule is probably responsible for the suppression of *BAK1* since priming occurred even though the bacteria were heat killed. It seems that *Bacillus* priming efficiency is not compromised

in *bak1-4* mutants, but the defense response was not effective. This suggests the possibility of other unidentified receptors playing a role in *Bacillus* mediated priming. Priming seemed to have an influence over plant development even though *BAK1* was absent, showing that other genes such as *BRI1* and *DET2* are regulated by priming and of importance. The mechanisms are not fully understood and further research is needed to elucidate the impact of MAMP signaling during beneficial bacterial mediated priming in plants.

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