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Role of Brassinosteroids in Plant Growth Promotion and Stress Tolerance During Priming Mediated by Beneficial Bacteria

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Brassinosteroiders roll i stimulering av tillväxt och stress tolerans hos växter efter priming med nyttiga bakterier

Role of Brassinosteroids in plant growth promotion and stress tolerance during priming mediated by beneficial bacteria

by

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Key words: Brassinosteroids, *Bacillus amyloliquefaciens*, priming, *Arabidopsis thaliana*

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ABSTRACT

Brassinosteroids (BR) are plant hormones widely distributed throughout the plant kingdom in low concentrations and with structural homology to animal and insect steroids. BR are involved in numerous physiological processes, and they also fulfill an antagonistic role in anti-herbivory structure formation in tomato (Campos *et al.*, 2009). In order to characterize the role of BR upon priming with *B. amyloliquefasciens* 5113, gene expression analysis of BR genes was assessed in *Arabidopsis thaliana*. *BAK1*, *BRI1* and *DWF1* expression down-regulates, while *DET2* up-regulates upon bacterial priming. *CPD* gene expression was not affected by priming. qPCR analysis of *VSP2* and *PR1* were performed on BR mutants upon priming with *B. amyloliquefasciens* 5113. Basal levels of PR1 were higher in *det2*, *bak1* and *dwf1* compared to primed samples. Primed *bri1* displayed two-fold higher expression of *PR1* compared to untreated *bri1*. *VSP2* level goes up on *det2*, *bak1* and *bri1* upon priming. No changes of *VSP2* expression were observed in *dwf1* upon priming. Methyl jasmonate treatment up-regulates *VSP2* level two-fold in *det2* and nine-fold in *bak1*. The role of BR genes in response to insect attack was examined. BR genes appear not to be responsive to herbivory by *S. littoralis*. However, *S. littoralis* larvae fed more on BR mutants compared to those that fed on Col-0 WT. In order to understand the role of BR in JA signaling pTRV-*JAR1* and pTRV-*LOX2* constructs were developed and virus induced gene silencing were performed on Col-0 and BR mutants *bak1* and *det2*. Gene silencing was confirmed by qPCR analysis of the target genes in Col-0 and *det2*, but not in *bak1*. Further insect feeding experiments are required to elucidate if BR play a role in defense responses to herbivory when JA signaling pathway is compromised.

BACKGROUND

Brassinosteroids (BR) are plant hormones widely distributed throughout the plant kingdom in low concentrations and with structural homology to animal and insect steroids. BR were discovered after about 10 years of research, where only 40 mg of pure Brassinolide were finally isolated from 40 kg of rapeseed pollen (Mandava, 1988) in order to determine the structure. Initial interest in BR was based on the growth-promoting properties of pollen extracts (Bishop and Koncz, 2002). The identification of genes involved in BR's perception and biosynthesis, including *CPD*, *DET2* and *BRI1,* and the loss-of-function mutants of those genes allowed the recognition of BR as a novel type of phytohormone in the 1990's due to severe growth defects typical of these mutants (Li *et al.*, 1996).

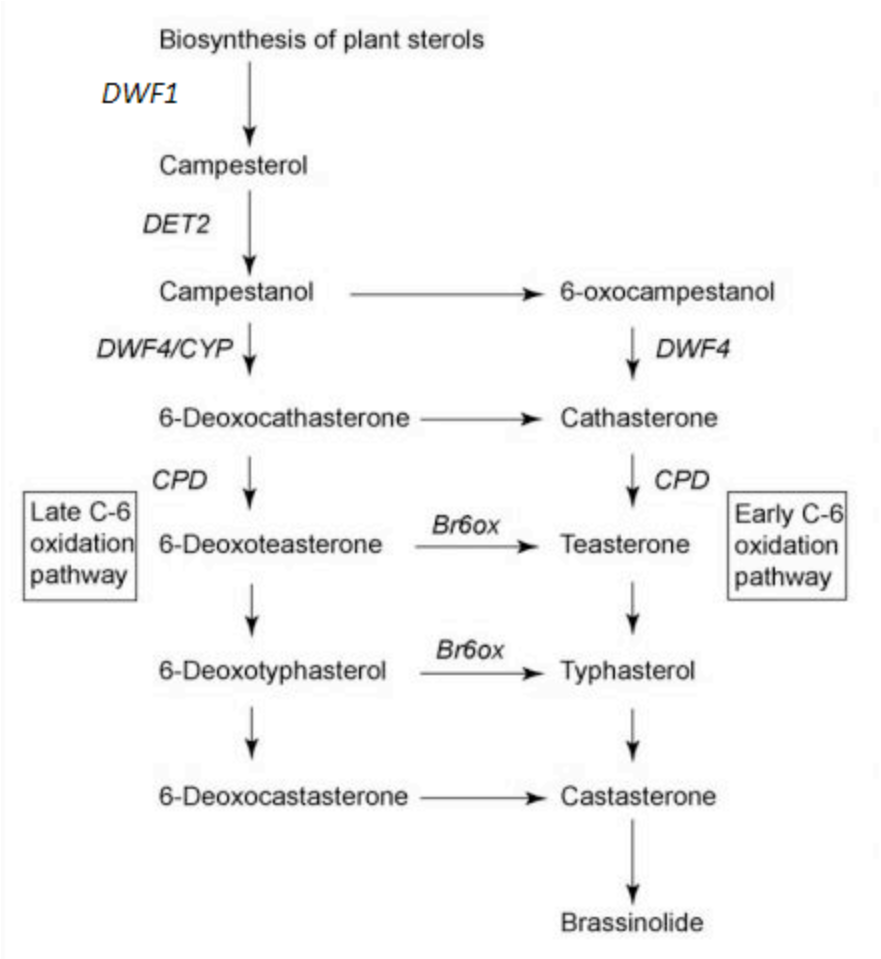

Figure 1. Brassinosteroid biosynthesis pathway. Taken from Divi and Krishna, 2009.

Till date about 50 naturally occuring BR have been identified, brassinoloide being the most active compound. The precursor of BR is campesterol, which is transformed into castasterone and subsequently into brassinoloide through early or late C-6 oxidation pathways (Figure 1) (Yang et al., 2011). Identification of genes involved in BR biosynthesis was made possible by molecular, genetic and biochemical analysis of dwarf mutants compromised in synthesis or perception of BR. *CPD* encodes a 23α-hydroxylase which is involved in the synthesis of 6-deoxoteasterone and teasterone (Szekeres and Koncz, 1998). *DET2* encodes a protein similar to mammalian steroid 5α-reductase which is able to metabolize human steroids when expressed in human kidney cells. Mutations in *DET2* compromise endogenous levels of BR (Li and Chory, 1997). *DWF1* is a calcium-dependent binding protein that encodes a sterol **Δ** 24 reductase involved in the conversion of 24-methylenecholesterol to campesterol (Kang *et al.,* 2001).

BRs are perceived by the plasma membrane receptor BRI1, which is an essential component to regulate the processes driven by BR (Figure 2). The BRI1 receptor has an extracellular domain with 24 leucine-rich repeats and an island domain. The BRI1 cytoplasmic domain contains a serine/threonine kinase domain, a juxtamembrane region and a C-terminal region (Li and Chory, 1997). The BAK1 receptor is also part of the perception complex, though it does not interact with BR, but is required for BRI1 activation via transphosphorylation (Wang *et al.*, 2005). BAK1 has 4 leucine zippers and 5 leucine-rich repeats in the extracellular domain along with a kinase domain and a C-terminal extension (Kim and Wang, 2010). Functional kinase domains of BAK1 and BRI1 are needed for mutual transphosphorylation. An intact extracellular domain of BRI1 is necessary but not sufficient for interaction with BAK1 but BRI1 kinase activity is also required for BR-induced association with BAK1.

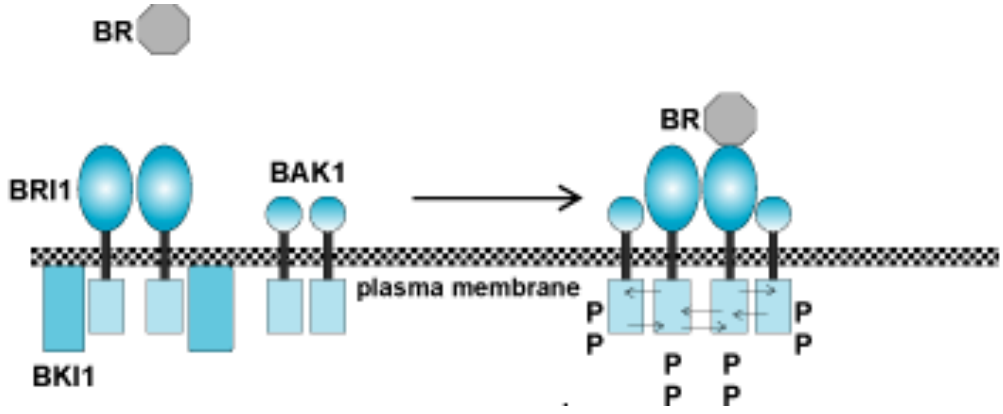

Figure 2. Brassinosteroid receptor complex. Taken from Yang *et al.*, 2011.

BR are involved in numerous plant processes such as: cell expansion and division, seed germination, xylem differentiation, reproductive development, pollen elongation and pollen tube formation. Furthermore, exogenous applications of BR have led to a broad spectrum of disease resistance (Buchanan *et al.*, 2000). Khripach and colleagues (2000) reported beneficial effects of BR on potato plants concerning plant productivity and susceptibility to *Phytophtora infestans.* The BR receptor BAK1 has been shown to interact with receptors that recognize pathogen-associated molecular patterns (PAMP), which triggers defence responses apparently independent of BR signaling (Chinchilla *et al.*, 2007). Apparently BR are also involved in responses to insects and might affect the development of anti-hervibory structures in tomato (Campos *et al.*, 2009). BR play an antagonistic role in the formation of trichomes by inhibiting jasmonic acid (JA) biosynthesis or signalling pathways, although the mechanism underlying the inhibition remains unknown.

BR play also a role in plant adaptation to environmental stresses including heat, cold, drought and salinity. The promotion of stress tolerance by BR is correlated with up-regulation of stress marker genes such as *RD29A* and *ERD10* (Dhaubhadel *et al.*, 1999), but the mechanism by which BR regulates the expression of these genes is still unknown. The NPR1 gene seems to play a central role in BR-mediated heat tolerance since the *npr1-1* mutant was not positively affected when sprayed with the synthetic BR analogue 24-epibrassinolide (EBR) (Divi *et al.*, 2010). Although it has been documented that BR interact with ethylene (ET), abscissic acid (ABA), gibberelic acid (GA) and JA in terms of growth regulatory processes, little is known in terms of genes involved in BR interaction with other hormones.

Bacillus amyloliquefaciens is a plant growth promoting bacterium (PGPB) that is capable of improving plant development by increasing the production of plant hormones or the availability of minerals and nutrients (Bloemberg and Lugtenberg, 2001). The genus *Bacillus* groups facultative and obligate aerobes which are characterized by their rod shape and ability to form oval endospores. They live in soil and some of them colonize the plant rhizosphere (Reva *et al.*, 2004). Several species produce antibiotics and others are able to mediate protection against pathogens and insects on plants, including *B. amyloliquefasciens*. The *B. amyloliquefasciens* 5113 strain was originally isolated from soil and can colonizeoilseed rape (*Brassica napus*) and

concedes protection against pathogens such as *Alternaria brassicae*, *Botrytis cinerea*, *Leptosphaeria maculans* and *Verticillium longisporum* (Danielsson *et al.*, 2007).

Bacteria such as *Streptomyces*, *Pseudomonas* and *Bacillus* can also enhance plant responses to pathogens and herbivores by priming a plant defense system called induced systemic resistance (ISR). Apparently PGPB prepares the plant to respond quickly to pathogens rather than triggering a constitutive defense response. ISR does not involve accumulation of salicylic acid (SA) or PR proteins connected with Systemic Acquired Resistance (SAR), but both metabolic pathways require functional *NPR1* for the response to occur (Bostock, 2005). ISR is usually regulated by JA and ET signalling pathways and potentiates broad defense responses to insects and pathogens. *B. napus* plants treated with *B. amyloliquefaciens* 5113 were less affected by *B. cinerea* compared with the untreated plants (Sarosh *et al.*, 2009). Transcript profiling revealed changes in expression of genes involved in metabolism, bioenergy and disease resistance. A significant up-regulation of BR regulated genes in the below ground and above ground tissues primed with beneficial bacteria was observed (Sarosh *et al.*, 2009).

AIMS

1. To characterize the role of BR responsive genes in enhanced plant growth upon *B. amyloliquefaciens* (5113) mediated priming.
2. Elucidate the role of BRs in stimulating the JA/SA responses in plants primed with *B. amyloliquefaciens*.
3. To define the role of BR in plant tolerance against *Spodoptera littoralis* herbivory.

METHODS

PLANT GROWTH CONDITIONS

Seeds of *Arabidopsis thaliana* wt Col-0 along with the BR mutants on Col-0 background *det2-1*, *dwf1-4*, *bak1-4* and *bri1* were put out on soil-vermiculite mixture without previous surface sterilization. One week after germination, seedlings were placed into single pots in order to improve growth conditions. Light conditions (16h/8h, L/D; ~ 200 μmol m^{-2} s^{-1}) and temperature (20°C) were controlled in a growth chamber.

Bacillus amyloliquefaciens 5113 GROWTH CONDITIONS AND PLANT INOCULATION

Bacteria were grown overnight in LB medium at 28°C and 180 rpm. Subsequently, the culture was transferred into 200 mL LB under the same temperature and agitation conditions for 24 hours. The bacterial culture was then centrifuged at 5000 rpm for 10 minutes in order to concentrate the bacterial cells. The pellet was resuspended in sterile water until reaching OD_{600} of 0.5. Three-week-old plants of Col-0, *det2*, *bri1* and *bak1* were supplied with 5 mL of the bacterial spore suspension ($1x10^7$/ml) via drenching. Control plants received 5 mL of water. Three days after inoculation leaves of primed and untreated plants were harvested for both insect feeding experiments and gene expression analysis.

INSECT FEEDING

Newly hatched larvae of *Spodoptera littoralis* were utilized to determine if there were any differences in feeding pattern when fed on Col-0 wt leaves compared to *det2*, *bri1* and *bak1* mutants. Leaves of three-week-old plants of the above mentioned genotypes were cut off and placed in separate petri dishes. Four larvae were placed in each petri dish and fresh leaves were given to the larvae every third day. The weight of the larvae was evaluated after 10 days.

RNA ISOLATION AND cDNA SYNTHESIS

Frozen leaves of three-week-old plants were pulverized using a bead beater. Subsequently, the ground tissue was used for RNA extraction following the protocol I of E.Z.N.A. Plant RNA Kit (OMEGA bio-tek). RNA concentration and purity were estimated by using a Nanodrop spectrophotometer. Total RNA (1 μg) was utilized for cDNA synthesis using a qScript cDNA

synthesis Kit (Quanta BIOSCIENCES). Purity and concentration of cDNA was also measured using a Nanodrop spectrophotometer.

RT-PCR ANALYSIS

CPD (**AT5G05690 forward primer** 5'-TGAAACAACCTCCACGATCA-3' and **reverse primer** 5'-TGCCCTAATCTTTTCATGCTCT-3'), *DET2* (**AT2G3850; forward primer** 5'-GTAAAAACGGATTTCCGATCAC-3' and **reverse primer** 5'- TGRAATGCGAAACCCAC CTC-*3'*), *BAK1* (**AT4G33430; forward primer** 5'-GGAATCAGAATTCTATCCGGGTGT-3' and **reverse primer** TTTGAGAGATCCAGAACTTGTAGC), *BRI1* (**AT4G39400; forward primer** 5'-AATTTCTCCGGTCCGATTCT-3' and **reverse primer** 5'-CTCCTGCAGAGTGT TTTTAGGG-3'), *DWF1* (**AT3G19820; forward primer** 5'-GCACTCGAATGGGTCCAC-3' and **reverse primer** 5'-TCCTTGTCTGTTTTCGTACTCG-3'), and *VSP2* (**At5g24770; forward primer** 5´-GTTAGGGACCGGAGCATCAA-3´ and **reverse primer** 5´AACGGTCACTGAG TATGATGGGT-3') gene expression were assessed in *A. thaliana* wt Col-0and Col-0 primed with *B. amyloliquefaciens* 5113. Gene expression of BR related genes (*CPD, DET2, BAK1* and *DWF1*) and the JA marker gene (*VSP2*) was also evaluated in insect wounded leaves of *A. thaliana* wt Col-0 and compared to undamaged plants of the same genotype. Gene expression of the JA marker gene *VSP2* and the SA marker *PR1* (**At2g14610; forward primer** 5-TGATCCTCGTGGGAATTATGT-3`, **reverse primer** 5-TGCATGATCACATCATTACTTCAT-3) were evaluated in the *A. thaliana* mutants *det2*, *bak1*, *bri1* and *dwf1* when primed with *B*. *amyloliquefaciens* 5113 and under control conditions. *Tubulin (***At5g62700; forward primer** 5`-CGATGTTGTTCGTAAGGAAGC-3´and **reverse primer** 5`-TCCTCCCAATGAGTGACAAA-3´) was used as reference gene. qPCR reactions were performed with SYBR green fluorescence mix (Fermentas) and analyzed on an ABRIPRISM 7000 sequence detection system.

SUBCLONING OF *JAR1*

A 394 bp fragment of *JAR1* gene was amplified with primers containing restriction sites (**AtJAR1fwdKpnI** 5'-AAGGTACCTTTCAGTAGAATCGGCTGC-3' and **AtJAR1revBamHI** 5'-AAGGATCCTGTGCTGAAGTAGCTACTC-3') from total DNA of *A. thaliana* wt ecotype Columbia. Life Pro Thermal Cycler (BIOER) was used to amplify the target

fragment with the following PCR conditions: denaturation at 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds and annealing at 50°C for 30 seconds. Extension was performed at 72°C for 11 minutes. The PCR product was loaded on a 1% agarose gel.

The fragment was cut out and DNA purified by using a Gene Jet Gel DNA extraction Kit (Fermentas) before ligation into PCR TOPO 2.1 vector (Invitrogen) following the TA Cloning Kit protocol (Invitrogen). Then, 100 µl of competent *E. coli* XL blue cells were added into 10 µl of ligation mixture and kept on ice for 20 minutes prior to heat shock at 42°C for 45 seconds followed by one minute in an ice bath to reduce cell damage. LB medium (200 µl) was added and the tubes were kept at 37°C for 1 h. LB plates containing ampicillin (100 µg/ml), x-gal (40 µg/ml) and IPTG (0.1mM) were used for blue/white selection of *E. coli* transformant colonies.

CLONING OF *JAR1* INTO TRV2 VECTOR

Positive colonies of *E. coli* were grown overnight in LB medium with ampicillin (100 µg/ml) before plasmid extraction. Overnight cultures were centrifuged for 10 minutes at 5000 rpm. A Gene Jet Plasmid miniprep kit (Fermentas) was used for plasmid extraction. In order to excise the target fragment of *JAR1* gene, 1 µg of plasmid was digested by adding 1 µl each of fast digestion enzymes *Bam*HI and *Kpn*I and 4 µl of TANGO buffer (Fermentas). The sample was gently mixed and kept at 37°C for 1 hour. The reaction was then stopped at 65°C. The digestion product was loaded on an 1% agarose gel. The band with the *JAR1* fragment was cut out and the DNA was extracted using a Gene Jet Gel DNA extraction Kit (Fermentas). Digestion of the plasmid TRV2 and purification of the linear TRV2 from the gel was performed using the same protocol mentioned above (Liu et al., 2002). In order to ligate the JAR1 fragment into TRV2, 15 ng of purified target fragment was added to 75 ng of linear TRV2 along with 1 µl of T4 DNA ligase and 2 µl of ligation buffer. Samples were carefully mixed and kept overnight at 14°C. The ligated vector was inserted into XL blue *E. coli* cells following the protocol previously described in the subcloning. The transformation mix was streaked on LB plates containing kanamycin (50 µg/ml) in order to select positive colonies, which were grown overnight in LB medium with kanamycin (50 µg/ml) for plasmid extraction. *Agrobacterium tumefaciens* GV3101 competent cells (100 µl) were mixed with 1 µg of the modified TRV2 vector and kept on ice for 5 minutes followed by 5 min in liquid nitrogen and 15 min at 37°C. The transformation mix was then

transferred into 1 ml LB medium at 28°C for 4 hours and 150 rpm. The culture was spun down at 5000 rpm for 2 min in order to pellet the bacterial cells. The pellet was resuspended in water. LB plates containing kanamycin (50 µg/ml) and gentamycin (15 µg/ml) were used for selection of positive colonies.

VIRUS INDUCED GENE SILENCING OF *JAR1* AND *LOX2* IN *Arabidopsis thaliana*

Transformed colonies of *A. tumefaciens* GV3101 containing TRV1 or TRV2-*JAR1, LOX2, PDS* or no insert - were grown following the protocol described by Caplan and Dinesh-Kumar (2006). In order to silence *JAR1* and *LOX2* genes, three week old plants of *A. thaliana* Col-0 wt along with the mutants *det2* and *bak1* were infiltrated with a solution with equal ratio of cultures of *Agrobactrium tumefaciens* GV3101 containing TRV2-*JAR1* or *LOX2*- and TRV1 in the abaxial side of two rosette leaves. In order to test the efficiency of the silencing system, the *PDS* gene was knocked down in the genotypes mentioned above and taken as positive control. As negative control, plants were infiltrated with *Agrobacterium* cells containing TRV2 vector without insert. Gene expression analysis was performed with *LOX2* and *JAR1* silenced plants to corroborate the down-regulation of the target genes. Insect feeding experiments were also carried out with *LOX2* and *JAR1* silenced plants following the protocol described previously.

METHYL JASMONATE TREATMENT

Three week-old plants of *A. thaliana* Col-0 wt, *bak1* and *det2* mutants were treated with either 30 uM methyl jasmonate (MJ) or water via spraying. Plants were kept in a cabinet with constant temperature (22°C) for two hours. Leaves of the treated plants were cut off and frozen with liquid nitrogen for RNA extraction and gene expression analysis as previously described.

RESULTS

BR GENE EXPRESSION UPON PRIMING WITH *Bacillus amyloliquefaciens*

Gene expression of BR biosynthetic and receptor genes were evaluated after priming with *B. amyloliquefaciens* 5113 and compared to constitutive expression in *A. thaliana* Col-0 wt (Figure 3). The BR receptor genes –*BRI1* and *BAK1*- showed down-regulation after priming, which might indicate that they are not involved in the response after priming. The *DWF1* gene showed the same trend as *BRI1* and *BAK1,* whereas a minor up-regulation was observed in *DET2* genes

after priming. No changes in gene expression of *CPD* were observed after bacterial priming. Since JA signaling is associated with bacterial priming, the *VSP2* gene was used as positive control, and showed 2.5 higher expression after priming.

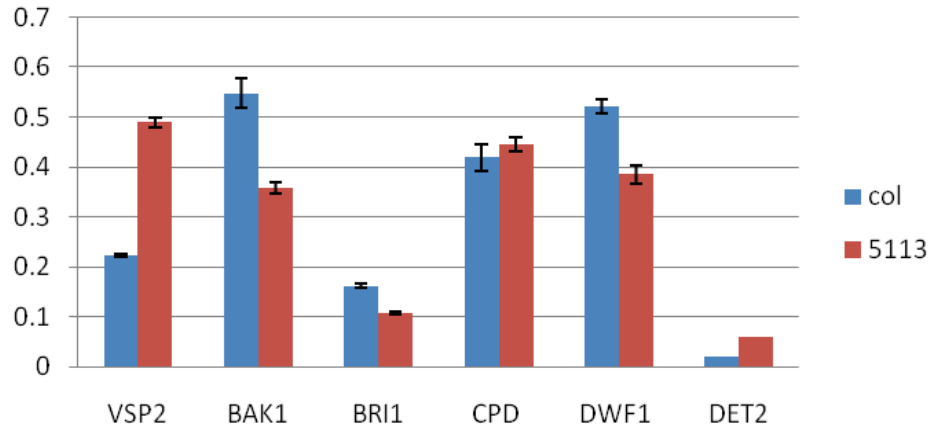

Figure 3. Brassinosteroid gene analysis upon priming with *B. amyloliquefaciens* 5113 in *A. thaliana* Col-0. The VSP2 gene was also analysed and used as positive control. Data was normalised and compared to a reference gene. X-axis normalised gene expression.

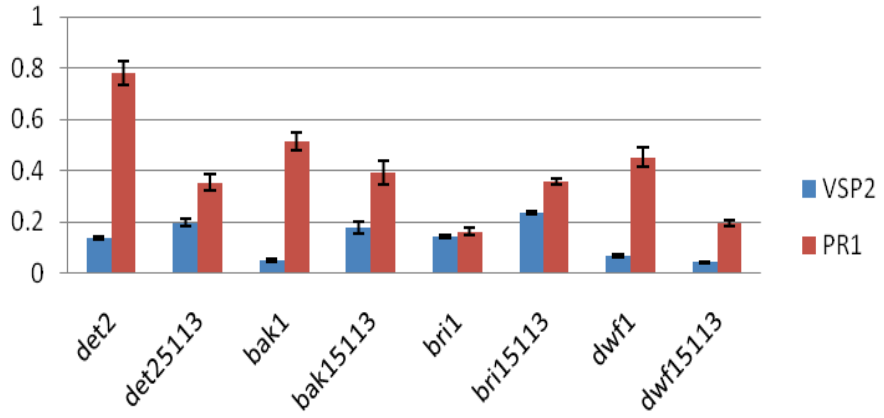

Figure 4. *VSP2* and *PR1* gene expression analysis in *A. thaliana* mutants *det2*, *bri1* and *dwf1* upon priming with *B. amyloliquefaciens* 5113 and control conditions. Data was normalised and compared to the reference genes Tubulin and Ubiquitin 5. X-axis normalised gene expression.

The effect of bacterial mediated priming on *VSP2* and *PR1* gene expression were investigated in the BR mutants *det2*, *bak1* and *dwf1* (Figure 4). Basal levels of PR1 were higher in *det2*, *bak1* and *dwf1* compared to the gene expression level upon priming. On the other hand, primed *bri1*

displayed two-fold higher expression compared to untreated *bri1*. Minor changes in *VSP2* gene expression were observed in the *dwf1* mutant upon priming, whereas *det2*, *bak1* and *bri1* showed higher expression in treated plants.

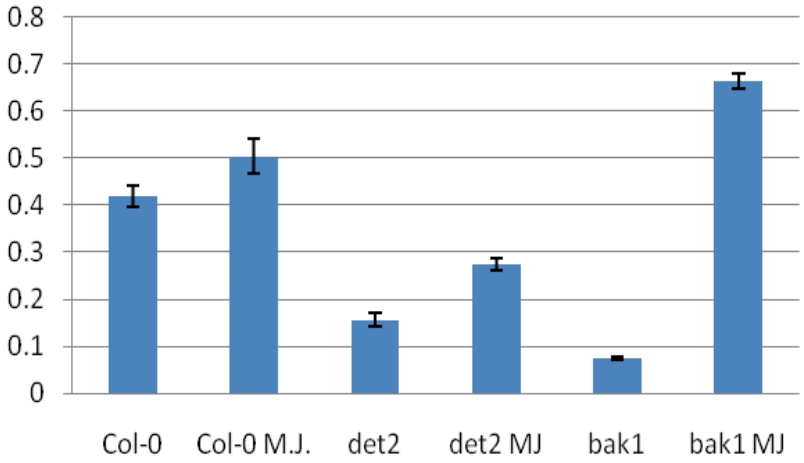

Figure 5. *VSP2* gene expression upon methyl jasmonate treatment in *A. thaliana* Col-0 wt and mutants *det2* and *bak1*. Data was normalised and compared to the reference genes (Tubulin and Ubiquitin 5). X-axis normalised gene expression.

MJ treatment up-regulated the *VSP2* gene in *det2* and *bak1* mutants (Figure 5). VSP2 was nine-fold upregulated in *bak1* after being treated, whereas *det2* showed two-fold higher expression when exposed to MJ. Col-0 wt displayed a minor up-regulation upon treatment compared to the BR mutants.

BR GENE EXPRESSION UPON HERBIVORY

BR gene expression was assessed in Col-0 wt upon herbivory with *Spodoptera littoralis* (Figure 6). No changes in gene expression of *BAK1* and *DET2* were observed when damaged leaves were compared to control treatment. *CPD* and *DWF1* genes showed down-regulation upon hervibory. JA is known to play an important role in mediating responses against herbivory. For that reason in the present experiment, gene expression of *the JA* marker gene *VSP2* was used as positive control. Up-regulation upon herbivory was observed as expected.

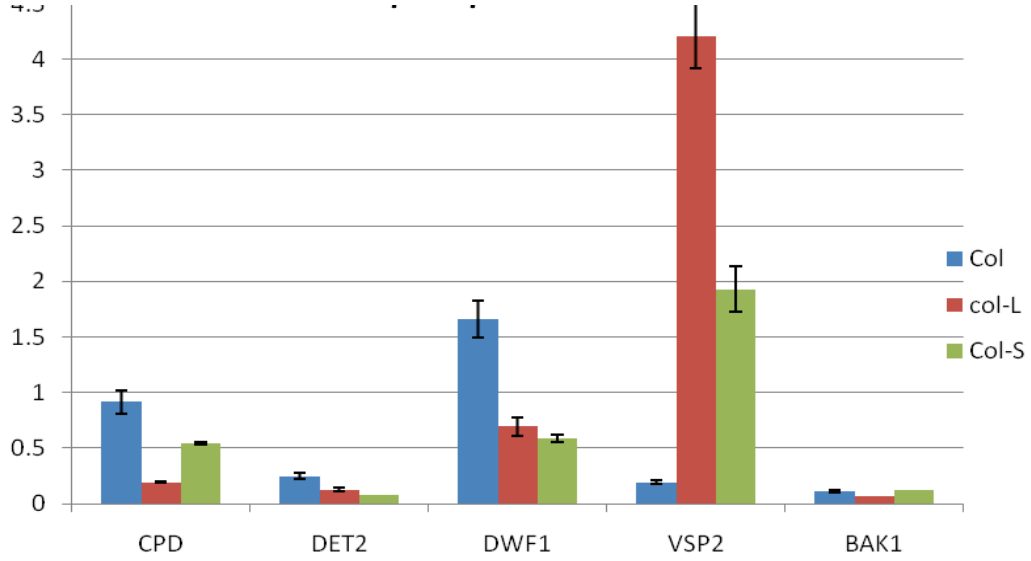

Figure 6. Brassinosteroid gene expression analysis in *A. thaliana* Col-0 wt upon insect feeding (*Spodoptera littoralis*). Expression was analysed in unwounded plants (Col), damaged leaves (Col-L) and in undamaged leaves of wounded plants (Col-S). Data was normalised and compared to two reference genes (Tubulin and Ubiqutin 5). X-axis normalised gene expression.

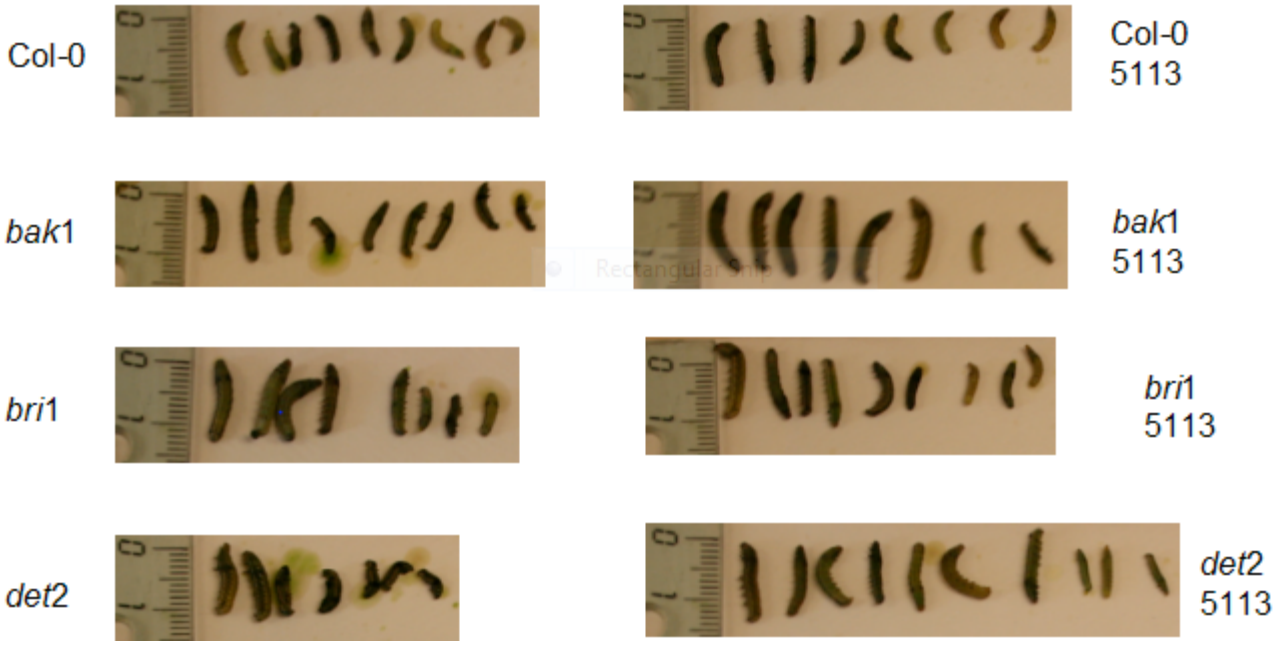

Figure 7. Non-choice insect feeding experiment with *Spodoptera littoralis* on *A. thaliana* Col-0 wt and mutants *bak1*, *bri1* and *det2* upon priming with *B. amyloliquefaciens* 5113 and control conditions. Leaves were collected 4 days after inoculation and placed on petri dishes along with newly hatched larvae. The weight of individual larva was checked after 10 days.

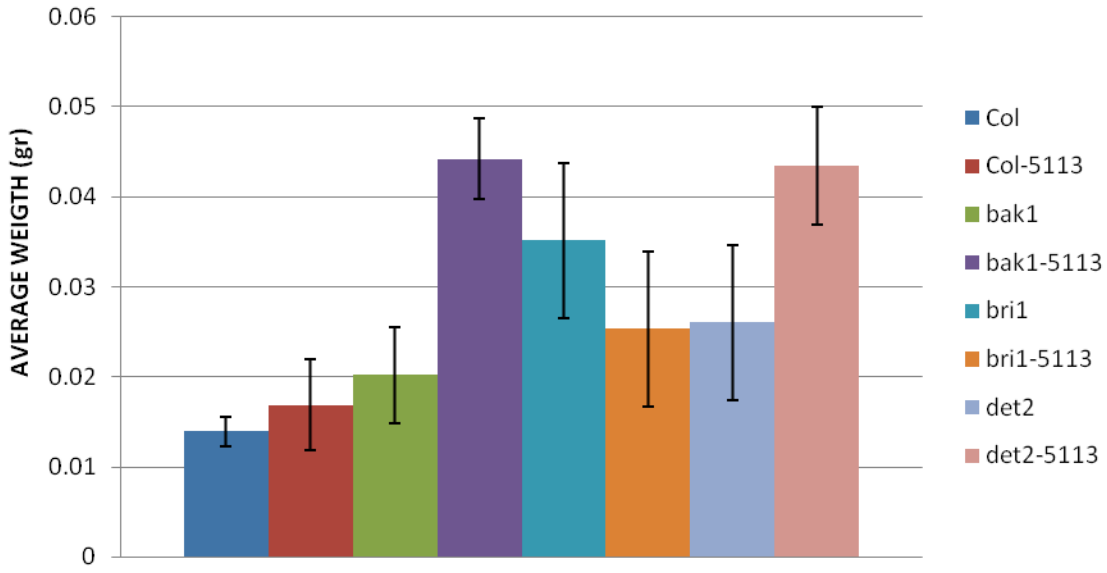

Figure 8. Statistical analysis of the non-choice insect feeding experiment. Average weight of larvae was used to calculate statistical differerences.

In order to elucidate if BR play a role in tolerance against hervibory upon priming with *B. amyloliquefasciens* 5113, newly hatched larvae of the generalist *S. littoralis* were placed on petri dishes containing leaves of primed and non-treated 3 week old plants of *A. thaliana* Col-0 wt and BR mutants *bak1*, *bri1* and *det2* (Figure 7). On the whole, larval weight within the treatments displayed high variability, so outlyers were discarded from statistical analysis. Individuals that fed on untreated *bak1*, *det2* and *bri1* were bigger compared to those that fed on untreated Col-0 WT. However, significant differences were seen only between untreated Col-0 , *bri1* and *det2*. Surprisingly, larvae feeding on primed Col-0, *bak1* and *det2* weighted more compared to those feeding on untreated leaves of the same genotypes. Statistical differences were also observed between *bak1* and *bak1*-5113 (Figure 8).

VIRUS INDUCED GENE SILENCING OF *LOX2* AND *JAR1* TO UNDERSTAND THE ROLE OF BR IN JA SIGNALLING

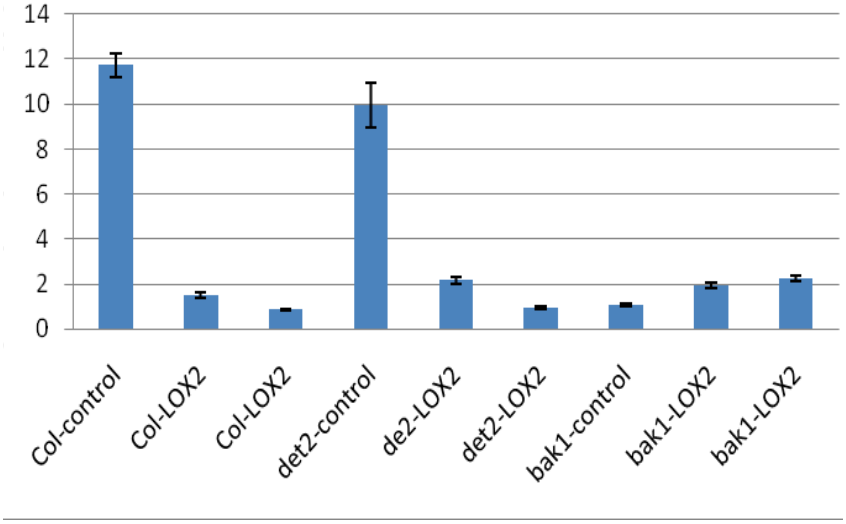

Figure 9. Gene expression analysis of *LOX2* gene in *A. thaliana* Col-0 wt and mutants *det2* and *bak1* upon virus induced gene silencing. Data was normalised and compared to the reference genes Tubulin and Ubiquitin5. X-axis normalised gene expression.

In order to corroborate the efficiency and specificity of the induced silencing experiment, qPCR analysis was preformed with systemic leaves of treated plants of Col-0 wt, *det2* and *bak1* 10 days after infiltration (Figure 9). *LOX2* gene expression was reduced by 85% in Col-0 and by 80% in the *det2* mutant, respectively. However no silencing was observed in the *bak1* mutant. Thr TRV-JAR1 construct efficiently silenced the *JAR1* gene in Col-0 and *det2*, with a reduction in gene expression of about 75% (Figure 10). No gene silencing of *JAR1* was detected in the *bak1* mutant either. An insect feeding assay was carried out to evaluate the effect of *LOX2* and *JAR1* silencing on defense responses against hervibory in Col-0 wt and the BR mutants *det2* and *bak1*. Unfortunately, due to high mortality of *S. littoralis* larvae in control treatments the data obtained from the experiment was not sufficient for statistical analysis.

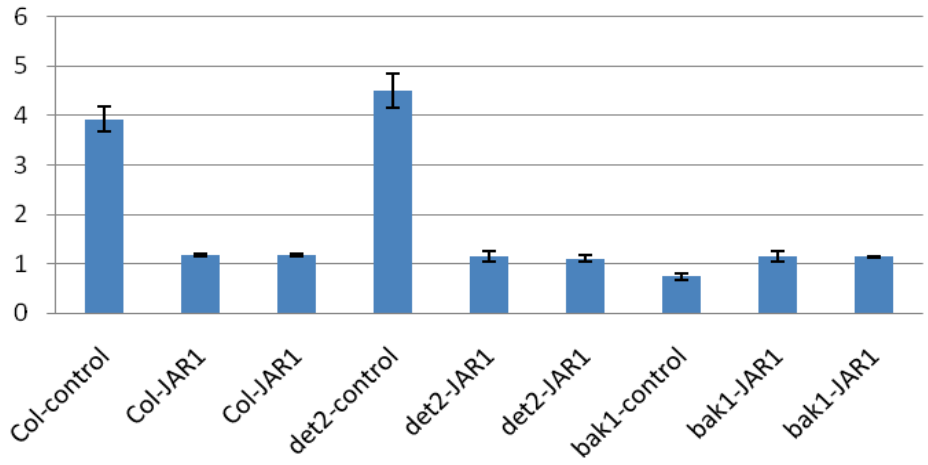

Figure 10. Gene expression analysis of *JAR1* in *A. thaliana* Col-0 wt and the mutants *det2* and *bak1* upon virus induced gene silencing. Data was normalised and compared to two reference genes (Tublin and Ubiquitin5). X-axis normalised gene expression.

DISCUSSION

Bacterial priming cause changes in gene expression. Sarosh et al. (2009) described up-regulation of transcript derived fragments (TDF) in *B. napus* upon priming with *B. amyloliquefasciens* 5113 and challenge with the pathogen *B. cinerea*. BLAST analysis of the TDF sequences allowed for identification of 16 homologues genes in *A. thaliana* which were involved in metabolism, signal transduction, energy production and transcription factors. Some of the homologues genes in *A. thaliana* are responsive to BR and MJ, including disease resistance genes.

However, when gene expression of BR related genes was analysed in *A. thaliana* down-regulation or no changes in gene expression was observed in four out of five genes. *DET 2* presented two-fold up-regulation after priming, although its constitutive expression was rather low compared to that of the other evaluated genes (Figure 3). Bacteria mediated priming brought about up-regulation of the JA marker gene *VSP2* as expected. According to the results obtained in the present study it appears that the analysed BR genes are independent of the JA pathway upon priming with *B. amyloliquefaciens* strain 5113.

In order to elucidate the role of BR related genes in priming, the *det2, bak1*, *bri1* and *dwf1* mutants were inoculated with *B. amyloliquefasciens* 5113 (Figure 4) and *VSP2* and *PR1* gene expression analysed. *det2*, *bak1* and *bri1* displayed elevated expression of *VSP2* upon priming

suggesting that these genes were not required for the JA mediated response upon bacterial priming. On the other hand, treated *dwf1* showed a minor down-regulation in gene expression of *VSP2* upon priming. The *dwf1* mutant displayed a 2-fold down-regulation of *PR1* upon priming as expected, since *PR1* is a SA marker and SA is not accumulated under induced systemic resistance (Choudhary *et al*, 2007). However, in order to corroborate if the *DWF1* gene is involved in the JA or SA pathways, qPCR analysis of the *VSP2* and *PR1* genes would be carried out on *dwf1* mutant upon either MJ or SA treatments.

Yang et al. (2010) demostrated that the Na*BAK1* gene in *Nicotiana attenuata* is involved in response to hervibory and acumulation of JA, even though it is not involved in the transcriptional regulation of JA. It seems it is not the case in *A. thaliana,* since the qPCR analysis revealed no changes in *BAK1* gene expression upon hervibory (Figure 6). Besides, when the *bak1* mutant was treated with MJ (Figure 5), a 6-fold up-regulation of *VSP2* was observed. That might indicate that there is an antagonistic role between *BAK1* and the JA pathway. Campos and collegues (2009) found via mutant analysis in tomato that BR negatively regulate trichome formation through acting on the JA pathway. Probably BR are not required for defense response against hervibores in *A. thaliana* since all evaluated BR genes showed down-regulation upon hervibory (Figure 6). It would be also interesting to check the expression level of BR genes in JA mutants upon hervibory in order to discard a possible role of BR genes in defense when the JA pathway is down.

Different pathways are activated upon insect attact which allow the plant to fine-tune specific responses (Lorenzo and Solano, 2005). The SA pathway is stimulated upon pathogen infection and phloem feeding aphids and mites, whereas tissue-damaging insects upregulates the JA and ET pathways (Occhipinti *et al.*, 2011). Along with the above mentioned phytohormones, other molecules are induced by insect attact. Volatile organic compounds, phenolic compounds, terpenes and alkenals play a role as airborne signals and some of them can activate defense responsive genes via calcium influx, phosphorylation and the action of reactive oxygen species (Arimura *et al*, 2000). Perhaps BR mutants present a low level of these compounds, which make them more appetising for a generalist like *S. littoralis* (Figure 8). It is also still unknown if BR are involved in glucosinolate synthesis and if those mutants have a lower constitutive level of glucosinolates compromising the response to generalist insects.

VIGS assay will allow us to investigate the role of BR in insect triggered responses in *A. thaliana* when the JA pathway is down and elucidate if there is a cross-talk between BR and JA as previously reported in tomato (Campos *et al.*, 2009). For that purpose, it is needed to repeat the insect feeding experiment with *S. littoralis* larvae with silenced plants (Figure 9 and 10) and evaluate larvae weight in the treatments. It would be worth to assess the level of BR upon bacterial priming with *B. amyloliquefaciens* 5113 in JA mutants to find out if BR genes are up-regulated upon priming when JA signalling is compromised.

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