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Abstract

Biocontrol through beneficial bacteria-mediated priming can promote plant growth and increase resistance to biotic and abiotic stress. *Bacillus amyloliquefaciens* strain 5113 has been identified as a promising candidate for priming by increasing stress resistance and promoting growth in oil seed rape (*Brassica napus*). Recent findings suggest that *WRKY20* transcription factor and the oligosaccharide raffinose are involved in increased resistance upon *B. amyloliquefaciens* strain 5113-mediated priming. The present study aimed to further elucidate the role of *WRKY20* transcription factor in plant defense as well as the involvement of *WRKY20* and raffinose in plant defense upon *B. amyloliquefaciens* strain 5113-mediated priming. Also, the effect on plant starch metabolism upon priming was studied by GUS analysis of isoamylase (*AtISA1*, *AtISA2* and *AtISA3)* expression in promoter+target::GFP/GUS transgenic plants. Role of *WRKY20* transcription factor in plant defense was studied through gene expression analysis upon treatment with salicylic acid (SA) and methyl jasmonate (MeJA). In order to elucidate the role of *WRKY20* and raffinose response to *Pseudomonas syringae* DC3000 infection in primed and non-primed Arabidopsis *wrky20, rs2* and *rs6* mutants and wild-type Col-0 was studied. *WRKY20* was shown to be up-regulated by SA treatment and down-regulated upon MeJA treatment. The infection study revealed an increased resistance in primed wild-type Arabidopsis plants compared to non-primed while no priming effect could be seen for *wrky20*, *rs2* and *rs6* mutants. Based on these and previous findings, *WRKY20* appears to function as regulator in the JA and SA dependent signaling pathways. Also, raffinose appears to be essential to increase plant resistance through *B. amyloliquefaciens* strain 5113-mediated priming. GUS analysis of *AtISA1*::GFP/GUS, *AtISA2*::GFP/GUS and *AtISA3*::GFP/GUS showed a distinct up-regulation of *AtISA1* in primed plants indicating an increased starch production. Taken together, this study suggests *B. amyloliquefaciens* strain 5113 as a promising candidate for biological control.

Keywords: Priming, *Bacillus amyloliquefaciens*, *WRKY20*, raffinose, salicylic acid, jasmonic acid, isoamylase

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Introduction

Beneficial bacteria-mediated priming

Agriculture heavily relies on the use of pesticides and resistance breeding for disease control to reduce yield loss. Extensive use of pesticides is harmful to the environment and there is a growing demand for more sustainable and environmental-friendly methods of cultivation. Also, disease control through use of chemical pesticides and resistance breeding do not provide a broad spectrum defense. Instead, protection is only provided against a small group of or specific plant pathogens (Pieterse *et al.*, 2002). Beneficial bacteria-mediated priming of plants is a promising alternative to the conventional methods of plant disease control. Biological control through priming induce the plants own defense systems and thereby provide an increased resistance to a broad range of different biotic and abiotic stresses and in some cases promote plant growth (Zamioudis and Pierterse *et al.*, 2012).

Plant defense and induced systemic resistance (ISR)

During evolution, plants have developed several sophisticated methods to combat different kinds of stresses. A drastic, but effective method used by plants to stop microbial pathogen invasions is to sacrifice plants cells, causing necrosis at the site of infection, and thereby prevent further spreading of the pathogen. This process is known as the hypersensitive response (HR) and often involves recognition of pathogen avirulence proteins (*Avr*) by plant resistance genes (*R*) (Feys and Parker, 2000). Also, plants have complex signaling pathways which may increase the plants defense response systemically upon pathogen attack (Dong, 1998). These signaling pathways rely on the plant hormones salicylic acid (SA) or jasmonic acid (JA) and ethylene (ET). Which signaling pathway, SA-or JA/ET-mediated, becomes activated depends on the type of pathogen or stress the plant is subjected to (Ton *et al.*, 2002; Cheong *et al.,* 2002). Accumulation of SA is associated with systemic acquired resistance (SAR) which involves the activation of pathogenesis-related (*PR*) defense genes providing a broad-spectrum resistance (Ton *et al.*, 2002). The JA/ET signaling pathway is associated with plant wound response, e.g. herbivore attack, but has also been shown to induce defense related genes such as defensin *PDF1.2* upon necrotrophic pathogen attack (Hammond-Kosack and Parker, 2003).

Increased resistance through beneficial bacteria-mediated priming is often referred to as induced systemic resistance (ISR). ISR is elicited through an activation of parts of the plants own defense signaling pathways and thereby facilitating a more rapid activation of defense responses leading to an increased resistance (Van Loon *et al.*, 1998). However, many of the processes underlying ISR remain to be elucidated and the processes involved seem to be dependent on which bacterium is used for priming. As an example, ISR have primarily been studied and reported for different species and strains of *Pseudomonas* spp. and was for a long time believed to be SA independent. However, SA dependent ISR have been reported for several strains of *Bacillus* spp. (Kloepper *et al.*, 2004), indicating that the underlying processes of increased resistance through priming with beneficial bacteria are not universal.

Bacillus amyloliquefaciens strain 5113

ISR through beneficial bacteria-mediated priming has primarily been studied and reported for *Pseudomonas* spp. However, several *Bacillus* strains have also been shown to elicit an increased stress resistance in plants (Kloepper *et al.*, 2004). Bacteria of the genus *Bacillus* are rod-shaped soil-bacteria often found in the plant rhizosphere. *Bacillus* spp. are also endospore-forming which facilitates effective formulation and several commercial bio-control products have been developed (Schisler *et al.*, 2004). The present study focused on *Bacillus amyloliquefaciens* strain 5113 which has been shown to be an effective colonizer of the roots of oil seed rape (*Brassica napus*), barley (*Hordeum vulgare*) and *Arabidopsis thaliana* (Reva *et al.*, 2004). In *B. napus*, root-treatment with *B. amyloliquefaciens* strain 5113 has been shown to trigger a systemic gene expression in leaves (Bejai *et al.*, 2009) and also increase resistance to several different fungal pathogens (Danielsson *et al.*, 2007). However, most of the molecular mechanisms involved in the increased resistance displayed by plants primed with *B. amyloliquefaciens* strain 5113 are still unknown.

WRKY transcription factors and raffinose

WRKY transcription factors have been shown to be involved in the regulation of many different plant processes, e.g. biotic defense (Dong *et al.*, 2003), abiotic stress response (Cheong *et al.*, 2002) and leaf senescence (Guo *et al.*, 2004). For a long time the WRKY family was thought to be plant-specific but has now also been identified in the primitive eukaryote *Giardia lamblia* and the slime mold *Dictyostelium discoideum* (Zhang & Wang, 2005). In Arabidopsis, 74 transcription factors have been identified as members of the WRKY family (Proietti *et al.*, 2011). The WRKY transcription factors are named after the WRKY domain, containing the heptapeptide WRKYGQK, at the N-terminal in all members of the family. Based on the number of WRKY domains and zink-finger-like motif characteristics the family is divided into three groups, I, II or III (Eulgem *et al.*, 2000). WRKY TF's regulate gene expression by binding to W-boxes, (T)(T)TGAC(C/T), present in the promoters of many defense-related plant genes (Eulgem *et al.*, 2000). Much general knowledge has been acquired about the WRKY family but the specific roles of many of the WRKY transcription factors remains to be elucidated due to the size of WRKY family and the diversity of process these transcription factors are involved in. However, a recent study have identified *WRKY20*, member of group I, as one of the key regulators of plant defense and resource allocation in Arabidopsis plants by regulating the signaling pathways of salicylic acid (SA) and jasmonic acid (JA) (Johansson *et al.*, unpublished). In the same study, metabolomic profiles revealed that *wrky20* mutants of Arabidopsis accumulate a significant amount of raffinose compared to wild-type Col-0 plants. Also, expression analysis showed an up-regulation of raffinose synthase 2 (*RS2*) in Arabidopsis plants primed with *B. amyloliquefaciens* strain 5113 compared to non-treated plants. Raffinose and its precursor galactinol have been reported to play a key role in beneficial bacteria-mediated enhanced plant tolerance against pathogens through JA signaling (Cho *et al.*, 2010). Based on these findings, the role of *WRKY20* and raffinose in plant defense upon *B. amyloliquefaciens* strain 5113-mediated priming was pursued in this study. Response to pathogen infection was studied by challenging primed and non-primed Arabidopsis wild-type Col-0 as well as *wrky20* and raffinose synthase mutants (*rs2* and *rs6*) with the hemibiotrophic pathogen *Pseudomonas syringae pv. tomato* strain DC3000. The

Arabidopsis-*Pseudomonas syringae* interactions are well studied and have generated a greater understanding of many of the mechanisms underlying plant/pathogen interaction and defense responses (Quirino and Bent, 2003). Resistant plants show low levels of disease symptoms and prevent *P. syringae* multiplication by triggering a hypersensitive response at the site of infection. In susceptible plants no HR response is triggered and disease symptoms, like necrotic lesions and chlorosis, is displayed (Katagiri *et al.*, 2002). Infection by *P. syringae* DC3000 is primarily associated with a SA dependent defense responses (Demianski *et al.*, 2012), but elevated JA defense responses have also been shown to increase resistance (Pieterse *et al.*, 2002).

Starch metabolism

B. amyloliquefaciens strain 5113-mediated priming has been observed to promote plant growth, but the underlying processes remain to be elucidated. However, increased plant growth might suggest an increased starch synthesis. In plants, starch is stored in the form of insoluble granules made up by amylopectin (typically ~75%) and amylose (Zeeman *et al.*, 2010). Starch is the primary plant storage carbohydrate and the main component of most crops harvested for human and animal consumption. Therefore, the effect of *B. amyloliquefaciens* strain 5113-mediated priming on starch metabolism was studied by studying gene expression of the isoamylases *AtISA1*, *AtISA2* and *AtISA3*. *AtISA1* is associated with synthesis of amylopectin while *AtISA3* is involved in starch degradation. *AtISA2* is non-catalytic but has been shown to be co-expressed with both *AtISA1* and *AtISA3* (Li *et al.*, 2007)

Aim

The present study aimed to elucidate the processes involved in and effects of priming with *B. amyloliquefaciens* strain 5113. Based on earlier studies, the following questions were formulated:

1. What is the role of *WRKY20* in plant defense?
2. Are *WRKY20* and raffinose involved in plant defense upon priming with *B. amyloliquefaciens* strain 5113?
3. Is starch metabolism affected by priming with *B. amyloliquefaciens* strain 5113?

Material & Methods

General experimental procedures

Plant material and growth conditions

Seeds of Arabidopsis wild-type Colombia (Col-0) and mutants were grown in pots at 23°C in a growth chamber under a 16/8-h photoperiod with 60% humidity and a light intensity of 30 µmol m^{-2} sec^{-1}. The Arabidopsis mutants *wrky20* (N653842), *rs2* (N613663) and *rs6* (N676927) were procured from Nottingham Arabidopsis Stock Center, UK. The seeds of *AtISA1:*:GFP/GUS, *AtISA2*::GFP/GUS and *AtISA3*::GFP/GUS were kindly provided by Dr. Ling (Iowa State University, US). Seeds were sterilized by washing with 10% commercial bleach solution and a few drops of TWEEN 20 for 15 minutes, washed with 70 % ethanol for ~1 min, followed by repeated rinsing with sterile water. Plants were grown on 0.5 x MS-media (Murashige and Skoog, 1962) in petri dishes at 22^{o}C under a 16/8-h photoperiod.

Priming with *B. amyloliquefaciens* strain 5113

All studies on effects of priming were performed with *Bacillus amyloliquefaciens* strain 5113 spore suspension (1x10^{7}/ml). Preparation of spore suspension was carried out by growing *B. amyloliquefaciens* strain 5113 in LB medium for three days and heat shock treatment was given at 70^{o}C for 10 minutes. The spore suspension was quantified by performing a serial dilution. For plants grown in pots, priming was performed by root drench inoculation of plants with 1 ml of 1x10^{7}/ml spore suspension. Seedlings grown in Petri dishes were primed by applying 20 or 2 µl spore suspension (1x10^{7}/ml) per root-tip.

Pseudomonas syringae pv. tomato DC3000

Pseudomonas syringae pv. tomato strain DC3000 was used in all infection experiments. Bacteria were grown on Kings B medium (10 g Proteose peptone #2, 1.5 g anhydrous K_2HPO_4, 15 g glycerol and 5 ml sterile 1 M $MgSO_4$ per liter of H_2O). Bacteria suspension of 1x10^{6} colony forming units (CFU)/ml was made in 10 mM $MgCl_2$. Challenge inoculation was performed two days after priming with *B. amyloliquefaciens* strain 5113 and were carried out with a needleless syringe into two fully expanded leaves of three-week-old plants.

Quantitative real-time PCR

Quantifications and expression analysis was performed using Applied Biosystems 7000 Real-Time PCR system using the following parameters: 2 min at 50^{o}C, 10 min at 95^{o}C, 40 cycles of 15 s at 95^{o}C and 1 min at 60^{o}C in 96-well optical reaction plates. qRT-PCR reactions (20 µl) included 1X Maxima™ SYBR/ROX master mix (Fermentas), supplemented with 5 µM primers and 5 µl cDNA as template. Primers for *WRKY20* (At4g26640), *Ubiquitin5* (At3g62270), *P. syringae* (16S NCBI GeneID: 1182221) and *LOX2* (At3g45140) are summarized in table 1. Triplicates of each sample were used for each analysis and negative template controls were included for each primer pair (reaction mixture without cDNA). Melting curve analysis was performed to ensure identity of amplicons and specificity of the reactions. The data were analyzed by the comparative C_T method (Livak and Schmittgen, 2001) with PCR efficiency

correction. PCR efficiency was determined based on the slope of the standard curves. Gene expression levels obtained by qRT-PCR were normalized using the *UBQ5* as endogenous reference and mean standard error was calculated according to Schmittgen and Livak (2008).

Table 1. List of primer sequences used in the study.

	Forward	Reverse
UBQ5	5'-CGATGGATCTGGAAAGGTTC-3'	5´-ACTCCATGTTCTGCGGTCTT-3´
WRKY20	5'-TCCGACTTCGTTTTTGGAAT-3'	5'-CGAGGCTTGAACAAAGAACC-3
P. syringae 16S	5'-CAGCTCGTGTCGTGAGATGT-3'	5'-CACCGGCAGTCTCCTTAGAG-3'
LOX2	5´-CTTACCCGCGGATCTCATC-3´	5'-AGCTCCACAGGTTGCGTTAG-3'

GUS-staining

Each 1.0 ml staining solution contained: 830 µl H_2O, 100 µl 1 M $NaPO_4$ pH 7.0, 20 µl 0.5 M EDTA, 10 µl 10% Triton X-100, 20 µl 50 mM $K_3Fe(CN)_6$ and 20 µl 0.1 M X-Gluc (diluted in N, N-Dimethylformamide). The seedlings were immersed in staining solution and incubated at 37^{o}C in darkness overnight. Staining solution was removed and the samples were washed repeatedly with 70% ethanol until the tissue had cleared. Photographs were taken using a Canon EOS 30D digital camera.

Response to pathogen infection in *wrky20*, *rs2* and *rs6* mutants and wild-type Col-0 of Arabidopsis

The experiment was performed with two-week old wild-type Arabidopsis ecotype Col-0 and *wrky20*, *rs2* and *rs6* mutant lines. Priming was carried out by root drench inoculation with 1 ml of *B. amyloliquefaciens* strain 5113 spore suspension ($1x10^7$/ml) for 18 wild-type Col-0, 20 *wrky20*, 16 *rs2* and 20 *rs6* plants. Also, 20 wild-type Col-0, 20 *wrky20*, 18 *rs2* and 20 *rs6* plants were kept untreated. Primed and non-primed plants were kept in separate trays to avoid cross-contamination of *B. amyloliquefaciens* strain 5113 treatment and were challenge inoculated two days after priming. Plants were challenge-inoculated on two leaves per plant with *P. syringae* strain DC3000 suspension using needleless syringe. Disease incidence and effect on plant development was recorded one week after infection. The inoculated leaves were collected and DNA was extracted using Automated Nucleic Acid Extraction, Mole Genetics, Norway. Quantification of *P. syringae* was performed with qPCR as described earlier using primers for *P. syringae* 16S region (Tab. 1) and a standard curve constructed from 0.1 ng up to $6.4x10^{-6}$ ng pure *P. syringae* DNA as template.

Time course study of *WRKY20* expression in Arabidopsis wild-type Col-0

The experiment was set up to study the time course induction of *WRKY20* expression in wild-type Arabidopsis (Col-0) after treatment with methyl jasmonate (MeJA), salicylic acid (SA), and feeding by second instar larvae of the generalist herbivore *Spodoptora littoralis*. Three-week old plants were sprayed with 30 µM MeJA and 1 mM SA and placed in separate chambers to avoid cross-contamination of the treatments. Samples were collected 0 h, 15 min, 30 min, 1 h, 3 h and 6 h after hormonal treatments. *S. littoralis* treatment was performed by placing one larva of the second instar stage on each plant. Larvae damaged leaf samples from two plants were pooled together and collected after 1 h, 3 h, 6 h and 24 h. For each time-point samples from water treated and non-damaged Col-0 plants were collected as controls including a 0 h sample. All samples were flash frozen with liquid nitrogen immediately after sampling and stored at -20^{o}C. The samples were homogenized with Retsch® MM200 and RNA extractions were performed using E.Z.N.A.™ Plant RNA Mini Kit (Omega bio-tek) according to manufacturer's recommendation. cDNA was synthesized from 1 µg total RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). Expression analysis of *WRKY20* was performed by qRT-PCR as described earlier and the relative expression was calculated using *UBQ5* as endogenous reference. For samples subjected to feeding by *S. littoralis*, *LOX2* was used as a positive control to ensure plant response to insect attack. See table 1 for primer sequences for *UBQ5*, *WRKY20* and *LOX2*.

AtISA1, *AtISA2* and *AtISA3* expression in Arabidopsis primed with *B. amyloliquefaciens* strain 5113

Five one-week old seedlings of *AtISA1*::GFP/GUS, *AtISA2*::GFP/GUS and *AtISA3*::GFP/GUS were treated with 20 µl *B. amyloliquefaciens* strain 5113 spore suspension (1x10^{7} /ml) on each root tip and an equal amount of seedlings were kept untreated. Samples were collected one and five days after treatment and stained for GUS. The activity was repeated for *AtISA1*::GFP/GUS using the same procedure except that 2 µl *B. amyloliquefaciens* strain 5113 spore suspension (1x10^{7}/ml) was applied on each root tip. Samples from the repeated experiment were collected for GUS staining six days after treatment.

Results

WRKY20 expression in Arabidopsis is affected by hormonal treatments and feeding by generalist herbivore

Three-week old wild-type Col-0 plants were treated with MeJA, SA, or subjected to feeding by second instar larvae of generalist herbivore *S. littoralis,* to study the effect on *WRKY20* expression. Expression levels in treated plants were compared to *WRKY20* expression in mock treated plants. Both hormonal treatments and feeding by *S. littoralis* had an effect on *WRKY20* gene expression. MeJA treatment resulted in a 1-fold down-regulation after 15 min and a 2-fold after 30 min but then remained at a constant level up to 6 h post treatment (Fig. 1A). Upon SA treatment, *WRKY20* gene expression was up-regulated 2.25-fold after 3 h and 3-fold after 6 h (Fig. 1B). Feeding by *S. littoralis* did not affect the *WRKY20* expression during the first 6 h after initiation of feeding. However, a distinct 2-fold up-regulation was detected after 24 h of feeding (Fig. 1C). *LOX2*, a marker for JA signaling pathway and herbivory response showed an up-regulated expression upon feeding by *S. littoralis*. The expression of *LOX2* was up-regulated already after one hour and continued to increase for the rest of the time span of the study, except for a dip at 6 h (Fig. 1D). *LOX2* was still up-regulated after 6 h, as compared to the 0 h control, but the sample deviated from the overall trend of the other samples, probably due to deviating feeding rate of the larvae.

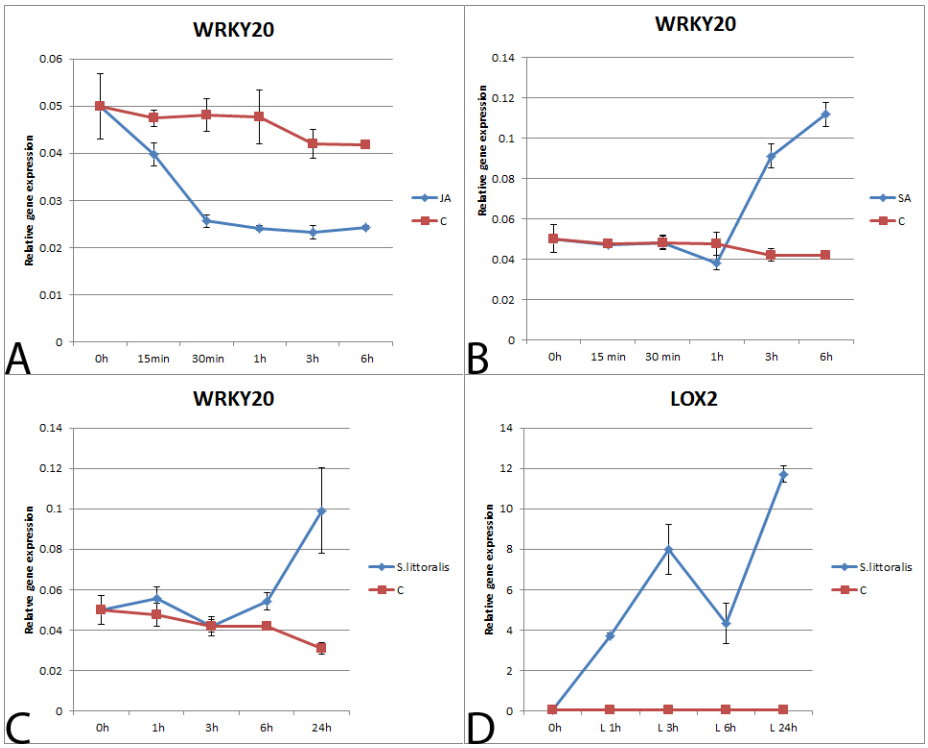

Figure 1. Relative gene expression of *WRKY20* calculated using *UBQ5* as endogenous reference, for plants treated with 30 µM JA (A), 1 mM SA (B) and subjected to feeding by *S. littoralis* (C). Up-regulation of *LOX2* expression in *S. littoralis* samples where included as a positive control for plant response to insect attack (D).

Bacillus amyloliquefaciens strain 5113-mediated priming increase resistance to *P. syringae* DC3000 infection in wild-type Arabidopsis

Untreated and primed Arabidopsis *wrky20*, *rs2*, *rs6* mutants and wild-type Col-0 plants were inoculated with *P. Syringae pv. tomato* strain DC3000. One week after inoculation, clear symptoms were observed in most of the leaves (Fig. 2). Spreading chlorosis and necrotic lesions could be seen on inoculated leaves for all lines and treatments. Non-primed wild-type Col-0 and *rs6* mutants as well as both primed and non-primed *rs2* mutants showed the most severe symptoms. Primed wild-type Col-0 and *rs6* mutants as well as primed and non-primed *wrky20* mutants appeared less affected by *P. syringae* DC3000 infection. Studying systemic symptoms, a clear priming effect was noticed for the wild-type Col-0 plants. Untreated Col-0 plants showed severe stress symptoms systemically while primed Col-0 plants appeared rather unaffected (Fig. 3). The results indicate that priming increases plant resistance to *P. syringae* in the wild-type Col-0. However, no priming effect could be seen for *wrky20*, *rs2* and *rs6* mutants. Systemic symptoms could be seen for both primed and non-primed *wrky20*, *rs2* and *rs6* mutants but *wrky20* plants displayed less severe symptoms compared to *rs2* and *rs6* mutants and non-primed Col-0 plants.

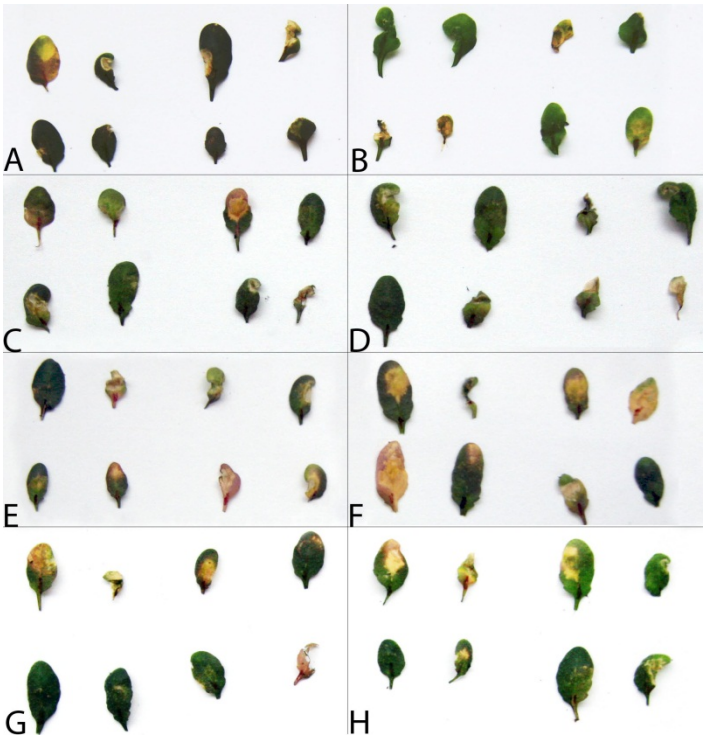

Figure 2. A representative selection of disease symptoms on inoculated leaves, one week after inoculation of *P. syringae* DC3000. A – Col-0 control, B – primed Col-0, C - *wrky20* control, D - primed *wrky20*, E - *rs2* control, F – primed *rs2*, G – *rs6* control and H – primed *rs6*.

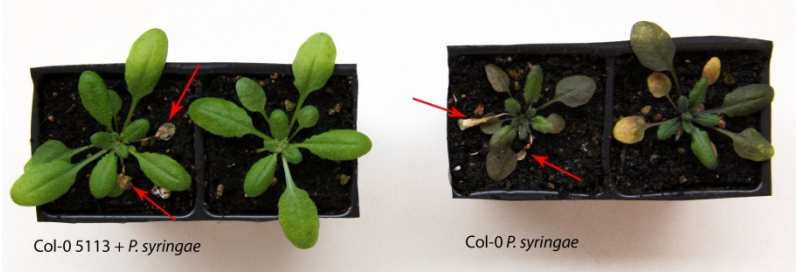

Figure 3. Representative picture showing the difference in systemic symptoms, one week after inoculation of *P. syringae* DC3000, for primed and non-primed Col-0. Red arrows indicate inoculated leaves. In the two plants without red arrows, inoculated leaves were sampled prior to photographing.

Priming with *B. amyloliquefaciens* strain 5113 inhibit *P. syringae* DC3000 growth in wild-type and *wrky20* Arabidopsis

Quantification of *P. syringae* in the inoculated leaves showed a lower concentration in *wrky20*, *rs2* and non-primed *rs6* plants compared to the wild-type Col-0 plants (Fig. 4). Primed wild-type Col-0 had a slightly lower pathogen concentration (7.1 ng/ml) compared to non-primed (9.2 ng/ml). A similar priming effect could be seen for the *wrky20* plants with 0.7 ng/ml in primed plants compared to 3.7 ng/ml for the non-primed. No priming effect was detected for *rs2* plants but the concentration for non-primed (2.4 ng/ml) and primed plants (2.6 ng/ml) were relatively low compared to the concentration in other samples. Pathogen quantification in *rs6* plants showed a much higher concentration in the primed plants (37.1 ng/ml) compared to non-primed *rs6* (4.9 ng/ml) and all other samples.

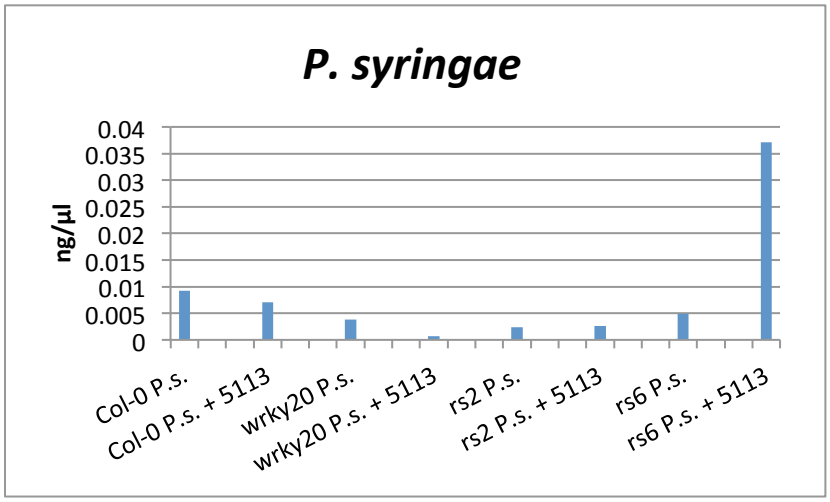

Figure 4. Quantification of *P. syringae* DC3000 in inoculated leaves of primed (5113) and non-primed *wrky20*, *rs2* and *rs6* mutants and wild-type Arabidopsis.

Priming affects bolting in Arabidopsis

When disease incidence was studied, a considerable difference in number of bolting plants was noticed between the different lines and treatments. At four weeks from planting, plants from all lines and treatments had started to bolt, except for primed wild-type Col-0 (Tab. 2). The most striking difference was between primed Col-0 plants and primed *wrky20* plants (Fig. 5), where 95% of the *wrky20* plants compared to 0% of the primed wild-type Col-0 plants had started to bolt.

Figure 5. Representative plants of wild-type and *wrky20* Arabidopsis primed with *B. amyloliquefaciens* strain 5113 and challenged with *P. syringae* DC3000.

Table 2. wrky20, rs2 and rs6 mutants and wild-type Arabidopsis plants bolting one week after inoculation of P. syringae DC3000.

Line	No. of plants bolting	
	Non-primed	Primed
Col-0	7(20)	0(18)
wrky20	14(20)	19(20)
rs2	11(18)	12(16)
rs6	14(20)	13(20)

Figures in parentheses indicate the total number of plants.

Priming with *Bacillus amyloliquefaciens* strain 5113 triggers an up-regulation of starch production in Arabidopsis

Difference in expression of *AtISA1*, *AtISA2* and *AtISA3* was studied in primed and non-primed Arabidopsis seedlings using GUS staining of promoter+target::GFP/GUS fusions in transgenic plants. A clear difference in *AtISA1* expression was seen between primed and non-primed seedlings (Fig. 6A and 6B). *AtISA1* expression was detected in all primed samples compared to no expression in the control samples. This suggests that *B. amyloliquefaciens* strain 5113-mediated priming triggers an up-regulation of starch synthesis. Expression was primarily seen in the leaves, but was also detected in the roots in the samples treated with 20 µl spore suspension per root tip. *AtISA1* plants primed with 2 µl spore suspension per root-tip showed a slightly lower expression in the leaves and no expression was detected in the roots (data not shown). *AtISA2* expression was high in both roots and leaves (Fig. 6C and 6D) while *AtISA3* expression was detected only in the leaves (Fig. 6E and 6F). No priming effect could be seen on the expression of *AtISA2* and *AtISA3*.

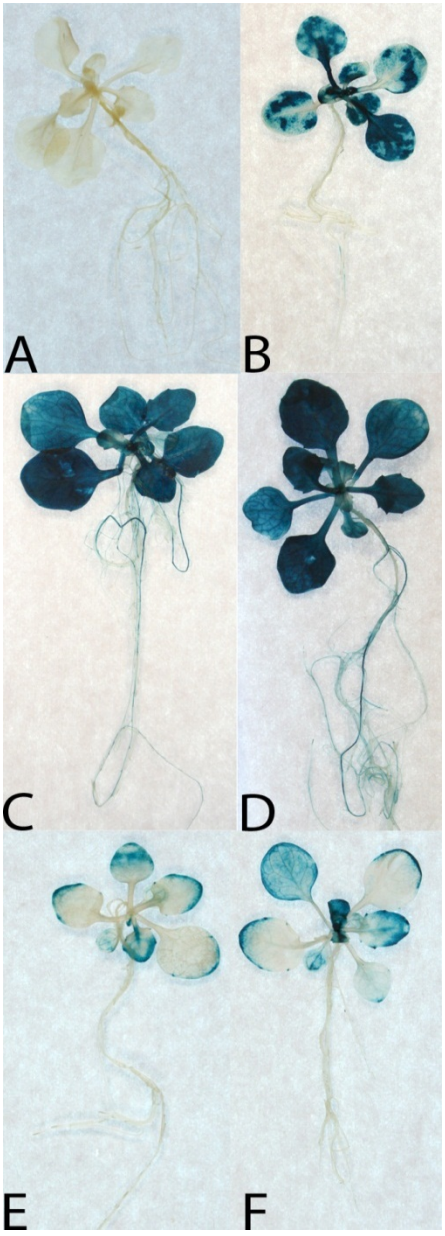

Figure 6. Representative samples from GUS staining of *AtISA1*::GFP/GUS, *AtISA2*::GFP/GUS and *AtISA3*::GFP/GUS one day after treatment. Primed plants were treated with 20 µl *B. amyloliquefaciens* strain 5113 spore suspension ($1x10^7$/ml) per root-tip. A – *AtISA1*, B – primed *AtISA1*, C – *AtISA2*, D – primed *AtISA2*, E – *AtISA3,* and F – primed *AtISA3*

Discussion

WRKY20 and Raffinose play a key role in plant defense upon *B. amyloliquefaciens* strain 5113-mediated priming

The time course study of *WRKY20* gene expression upon hormonal treatments indicated that SA treatment triggers an up-regulation of *WRKY20* expression, and JA cause a down-regulation. The results of the hormonal treatments correspond with Genevestigator data (Hruz *et al.*, 2008) for *WRKY20* (Fig. 7). Also, previous experiments have shown that *wrky20* mutants accumulate more raffinose (Johansson *et al.*, unpublished) which has been shown to be essential for JA-dependent defense responses (Cho *et al.*, 2010). Based on the results of the present study combined with the results of previous studies, a hypothesis for the role of *WRKY20* as a regulator of JA and SA defense responses is formulated (Fig. 8A). Dependent on which signaling pathway is activated upon pathogen attack, *WRKY20* is either up- or down-regulated, and determines if SA-or JA-responsive defense genes are activated. Present data suggest that a SA response triggers an up-regulation of *WRKY20* followed by activation of SA-responsive defense genes. Conversely, JA response down-regulates *WRKY20* followed by an up-regulation of raffinose levels and activation of JA-responsive defense genes. However, in the time course experiment an up-regulation of *WRKY20* was detected after 24 h of feeding by *S. littoralis*. Wounding of plants as well as herbivore attack are associated with JA mediated defense responses in plants (Dong, 1998) and up-regulation of *WRKY20* expression after *S. littoralis* is therefore unexpected according to the hypothesis previously stated. It should be noted that the feeding experiment was performed in a small scale and more studies are needed to fully resolve the role of *WRKY20* in plant defense signaling.

Figure 7. Genevestigator data (https://www.genevestigator.com) for *WRKY20* showing a down-regulation upon MeJA treatment and up-regulation upon SA treatment which corresponds with the results from the present study.

For wild-type Col-0 plants, priming clearly increased resistance to *P. syringae* DC3000 infection while no priming effect was detected in *wrky20*, *rs2* and *rs6* mutants. Raffinose and its precursor galactinol have been shown to be needed to induce JA mediated defense responses (Cho *et al.*, 2010) and previous experiments have shown that priming with *B. amyloliquefaciens* strain 5113 triggers an up-regulation of raffinose synthase 2 (*rs2*) (Johansson *et al.*, unpublished). Therefore, the lack of priming effect in the *rs2* and *rs6* mutants suggests that *B. amyloliquefaciens* strain 5113-mediated priming induces a JA-dependent resistance. Based on these findings and the hypothesis formed for the role of *WRKY20* in plant defense, a simplified model for the increased resistance in plants upon priming with *B. amyloliquefaciens* strain 5113 is proposed (Fig. 8B). According to this model the *wrky20* mutants would be expected to show a clear priming effect in the infection study since the raffinose levels are expected to be particularly high in the primed mutants. Quantification of *P. syringae* DC3000 in the inoculated leaves of the *wrky20* mutants indicated a priming effect, but this could not be seen when comparing the plant phenotypes. Both primed and non-primed plants showed systemic disease and stress symptoms but these were less severe compared to the other lines and treatments showing systemic disease symptoms. This might suggest that the *P. syringae* infection actually was suppressed in the *wrky20* mutants and that the observed stress symptoms might be an effect of completely silencing *WRKY20* expression. When studying the effects on plant development it was noticed that primed *wrky20* mutants had the highest proportion of bolting plants.

Early bolting can be a strategy for stressed plants to spread pass on their genetic material before they perish. As previously stated, raffinose is a part of the JA signaling pathway but has also been reported to have a key role in defense towards oxidative damage (Nishizawa *et al.*, 2008). It is possible that too high raffinose levels triggers high levels of stress responses in the plant leading to high cost and negative effects on plant fitness.

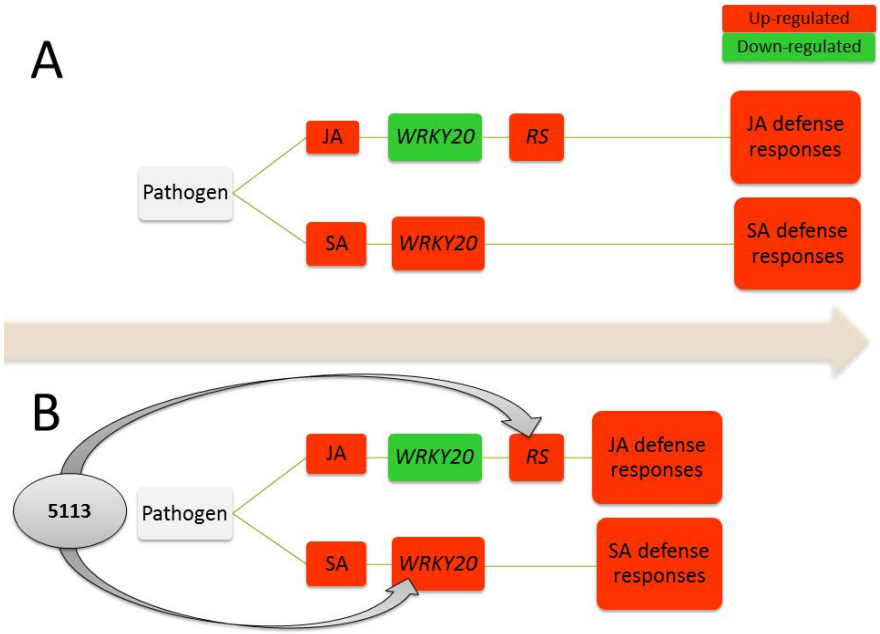

Figure 8. A – Pathogen elicits either SA or JA signaling leading to an up- or down-regulation of *WRKY20*. An up-regulation of *WRKY20* triggers the expression of SA responsive defense genes and a down-regulation of *WRKY20* triggers an up-regulation of raffinose synthases and expression of JA responsive defense genes. B – In primed plants, the basal levels of raffinose and *WRKY20* are higher resulting in a more rapid JA or SA responsive defense response and thereby increasing resistance.

More studies are needed in order to fully resolve the function of *WRKY20* in plant defense and the mechanism involved in ISR through *B. amyloliquefaciens* strain 5113 priming. To further pursue the role of *WRKY20* in JA and SA signaling pathway, infection studies involving *wrky20*/JA-marker and *wrky20*/SA-marker double knockouts is proposed. Also, effects on pathogen resistance upon *WRKY20* overexpression in combination with expression analysis of SA and JA marker genes and measures of SA and JA levels might provide significant evidence for the role of *WRKY20* transcription factor.

Priming with *B. amyloliquefaciens* strain 5113 up-regulates plant starch production

In plants primed with *B. amyloliquefaciens* strain 5113 *AtISA1* expression was induced while no expression was detected in non-primed plants. No difference in expression was detected for *AtISA2* and *AtISA3* between primed and non-primed plants. *AtISA1* has been shown to be involved in starch synthesis through involvement in the synthesis of amylopectin, and *AtISA3* is involved in starch degradation (Delatte *et al.*, 2005; Wattebled *et al.*, 2005). *AtISA2* lacks the necessary amino acid residues for catalytic debranching activity, but probably functions as a regulator or stabilizer in complex with both *AtISA1* (Delatte *et al.*, 2005) and *AtISA3* (Li *et al.*, 2007). *AtISA1* was expressed in all primed samples while expression could only be detected in one of the control samples. Seedlings were collected for staining early in the day and the lack of expression of *AtISA1* together with expression of *AtISA3* in the non-primed plants indicates that the plants were still respiring after the night. Priming with *B. amyloliquefaciens* strain 5113 did not affect the expression of *AtISA2* and *AtISA3* but induced expression of *AtISA1* in all samples. *B. amyloliquefaciens* strain 5113 priming probably speeds up the starch synthesis after respiration. However, starch degradation did not seem to be affected leading to simultaneous expression of *AtISA1* and *AtISA3*. To further investigate the effects of *B. amyloliquefaciens* strain 5113-mediated priming on starch metabolism it would be interesting to repeat the experiment, but collect samples during night to ensure that priming does not trigger a constant activation of *AtISA1* expression. Also, the study should be complemented with a starch content analysis of primed and non-primed plants. The results of this study indicate that plant primed with *B. amyloliquefaciens* strain 5113 probably produces more starch compared to non-primed, but the simultaneous expression of *AtISA1* and *AtISA3* requires further studies.

Concluding remarks

This study has presented evidence for an increased resistance to *P. syringae pv. tomato* DC3000 in Arabidopsis Col-0 primed with *B. amyloliquefaciens* strain 5113. In addition, *WRKY20* transcription factor and raffinose have been shown to play a key role in the induced systemic resistance (ISR) in primed plants.

Evidence was also provided that plants primed with *B. amyloliquefaciens* strain 5113 have an increased starch production which might be one of the factors in the promotion of plant growth observed in primed plants.

Lastly, this study confirmed *B. amyloliquefaciens* strain 5113 as a promising candidate to be used for biological control. Increased knowledge of the effects of and molecular processes underlying beneficial bacteria-mediated priming is essential in order to be able to develop a product for practical application in agriculture.

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