Biocontrol Elicited Systemic Resistance in Sugarbeet is Salicylic Acid Independent and NPR1 Dependent

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ABSTRACT

Systemic acquired resistance is an innate defense response in plants to a broad array of pathogens. Salicylic acid (SA) and non-expressor of pathogenesis-related genes1 (NPR1) are key players in systemic acquired resistance. Previously we demonstrated the biological control agent *Bacillus mycoides* isolate BmJ controls Cercospora leaf spot of sugarbeet through induction of pathogenesis-related (PR) proteins and biphasic hydrogen peroxide production. Both of these factors are associated with SA- and NPR1-dependent resistance induction. Here we show BmJ treatment leads to monomerization of NPR1, a phenomenon observed with activation of defense in *Arabidopsis*. The identity of the NPR1 monomer was confirmed using MALDI-TOF-MS. In contrast to the *Arabidopsis* system, monomerization of NPR1 in sugarbeet is not preceded by SA accumulation when triggered by BmJ. The SA levels following BmJ treatment were equivalent with water and *Bacillus pumulis* isolate BMH5E-33 treatments. BMH5E-33 is a non-inducer of resistance. The timeline of NPR1 activation by BmJ (3 hours post application) coincides with the previously observed secondary burst of hydrogen peroxide elicited in sugarbeet by BmJ treatment. These observations suggest PR-proteins induced by BmJ are induced through a SA-independent, NPR1-dependent pathway similar to that reported for the systemic resistance inducer

product, Actigard (acibenzolar-S-methyl [ASM], Syngenta, Greensboro, NC). Therefore the SA signal transduction cascade may be activated downstream of SA accumulation similar to ASM, or activation of NPR1 and PR-proteins may occur through a novel signal transduction pathway.

Additional Key Words: *Bacillus mycoides*, systemic acquired resistance, pathogenesis-related proteins, oxidative burst, biological control.

Plants have a variety of means of defending themselves against pathogen attack. Inducible broad-spectrum resistance provides long-term defense against a wide array of pathogens and has been described in several plant systems in response to a variety of stimuli (Ryals *et al.*, 1996; van Wees *et al.*, 1997; Yasuda *et al.*, 2003). Signaling components involved in defense are largely unknown. Important compounds that have been identified, include salicylic acid (SA) (Ollerstam and Larrson, 2003; Shapiro and Gutsche, 2003), jasmonic acid (JA) and ethylene (Heil and Bostock, 2002). All of these are considered secondary signal molecules. Downstream signaling components, such as non-expressor of pathogenesis-related gene1 (NPR1) are deployed during SA-dependent defense and orchestrate cross-talk between SA and JA pathways (Spoel *et al.*, 2003). The antagonistic SA and JA pathways elicit the accumulation of distinct subsets of defense-related proteins. SA-dependent pathways are associated with pathogenesis-related (PR) proteins such as chitinase, peroxidase, ß-glucanase and PR-1 (Durrant and Dong, 2004) all of which are associated with BmJ-induced resistance in sugarbeet effective against *Cercospora beticola* (Bargabus *et al.*, 2003). The JA and ethylene pathways are associated with production of thionins, defensins and proteinase inhibitors (Xu *et al.*, 2001).

Salicylic acid is tied to the oxidative burst, one of the earliest events in the establishment of induced resistance. Interaction occurs between SA and active oxygen species (AOS) produced during the oxidative burst. SA inhibits antioxidant enzymes causing an increase in AOS concentration (Durner and Klessig, 1995). Additionally, hydrogen peroxide stimulates two enzymes (Leon *et al.*, 1995) in one of the SA biosynthetic pathways. SA and AOS also are linked to activation of NPR1, a protein that functions downstream of SA signaling. NPR1 is constitutively expressed in an inactive multimeric state. Increased concentrations of AOS during the oxidative burst triggers overproduction of antioxidant enzymes in the plant. The AOS scavenging is hypothesized to create the reducing environment necessary to release active monomers of NPR1 (Mou *et al.*, 2003). The monomeric NPR1 moves to the nucleus, associates with TGA transcription factors and activates PR-genes (Fan and Dong, 2002) (Figure 1).

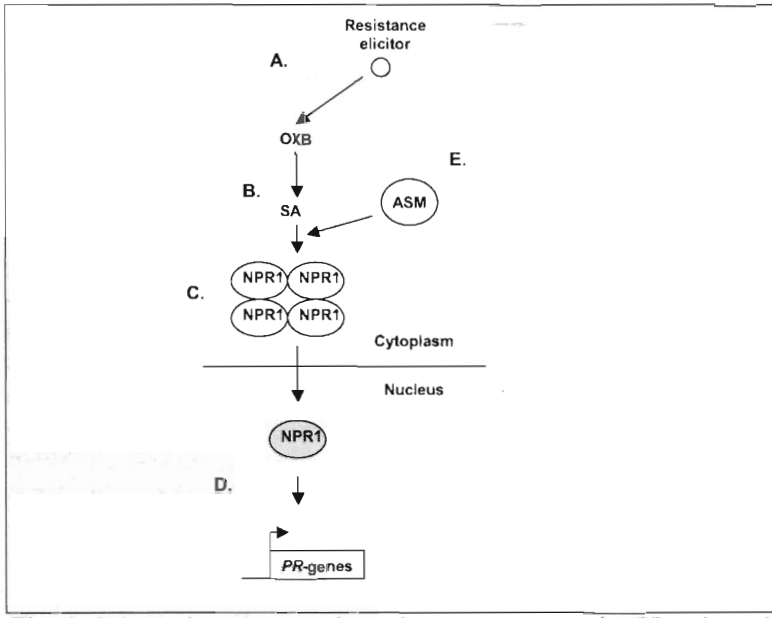

Fig. 1. Schematic representation of the activation of NPR1 through salicylic acid dependent signaling. A. Plant perception of a resistance elicitor is followed by a rapid accumulation of active oxygen species, known as an oxidative burst (OXB). B. The OXB stimulates accumulation of salicylic acid (SA) which acts upstream of the reduction of the inactive multimer of NPR1 found in the plant cytosol (C). D. The active monomer of NPR1 has an exposed nuclear localization signal, therefore the protein is translocated from the cytoplasm into the nucleus, where it interacts with transcription factors and activates transcription of pathogenesis-related genes (PR-genes). E. ASM, a functional analog of SA, can activate the same pathway, but downstream of SA accumulation.

Previously we described the biochemical outcome of treatment of sugarbeet with *Bacillus mycoides* isolate Bac J (BmJ) (Bargabus *et al.*, 2002). BmJ induces resistance that affords partial protection against *Cercospora beticola*, the causal agent of Cercospora leaf spot, a foliar pathogen of sugarbeet. BmJ induced resistance provides 70-91% control of Cercsopopra leaf spot on susceptible varieties and provides control equivalent to commonly used fungicides on more resistant varieties (Jacobsen *et al.*, 2004). Since the resistance is associated with production of SA-associated PR-proteins (peroxidase, ß-glucanase and chitinase), we hypothesize that the signaling pathway is SA dependent.

Due to the fact that BmJ elicits an oxidative burst (Bargabus *et al.*, 2003), we speculate NPR1 may also be deployed in the establishment of resistance. In the current investigation we examine levels of SA and the state of NPR1 following BmJ treatment.

MATERIALS AND METHODS

Plant Culture. *Beta vulgaris* germplasm 'FC 607' (PI 590837) (Smith and Ruppel, 1980) was seeded into 20-cm diameter pots containing pasteurized Metro-Mix 200 (The Scotts Company, Marysville, OH) supplemented with Osmocote 14-14-14 (The Scotts Company, Marysville, OH). Seed was dusted with a 4:1(v/v) charcoal/metalaxyl (Apron, Gustafson, Plano, TX) mixture prior to planting. Plants were maintained in a glasshouse at 22°C ± 5°C, were watered twice a week and kept under 12 to 14 hours of daylight. At about four weeks, imidacloprid (Marathon, 1% granular, 1 gram/pot) and triadimefon (Strike, foliar spray, 1.9g l-1) (Olympic Horticultural Products Co., Mainland, PA.) were used as preventatives for thrip/aphid feeding and powdery mildew infection, respectively. Plants used in all experiments were between 5 and 7 weeks of age.

Bacterial Cultures. *Bacillus mycoides* isolate Bm J, originally isolated from sugarbeet leaves in Sidney, Montana in 1994 (Bargabus *et al.*, 2002), was stored at -80°C in 10% glycerol and 1% tryptic soy broth (TSB; Difco, Sparks, MD). For fresh cell preparations, BmJ was cultured in TSB for 48 hours (28°C). Cells were harvested by centrifugation at 10,000 g (4°C) for 15 minutes. The cells were washed with sterile distilled water (2x) to remove any residual spent growth media. Following the washes the cells were resuspended in distilled water and the optical density was adjusted to A_{600} 1.0, then diluted 1:2. This yielded approximately 1 x 10^8 cfu ml^{-1}. The precise number of cells was not determined due to the chain forming nature of *B. mycoides*. *Bacillus pumulis* isolate BMH5E-33, originally isolated from the sugarbeet rhizosphere in Sidney, Montana in 1997, was prepared using the same protocol as employed with BmJ. For experiments testing killed cells, BmJ was autoclaved in water for 30 minutes following the washing procedure. Autoclaved cells were tested for lack of viability by plating 100 μl of the washed cells onto three plates of 50% tryptic soy agar, and confirmed by lack of colony formation at 28°C for 48 hours.

Sugarbeet Treatment. Acibenzolar-S-methyl (ASM, 50 ppm a.i.; Actigard 50WG, Syngenta, Greensboro, NC), a known chemical inducer of resistance, was applied as an experimental negative and positive

control for SA and NPR1 analysis, respectively. ASM, live or autoclave killed BmJ, or BMH5E-33 cells were individually spray applied to all fully expanded leaves until it began to pool and nearly drip off the leaf surface. Water was spray applied as an experimental negative control. In SA experiments, probenazole (PBZ; Dalian Indicom Agrichemicals, Dalian, China) an inducer of SA-dependent resistance was used as a positive control. In NPR1 experiments, SA (2 mM in 0.1M potassium phosphate buffer, pH 7.0, containing 0.01% triton x-100) was an additional positive control.

Salicylic Acid Detection. At each time point, one half of a treated leaf was excised and weighed (one leaf half per plant; two plants per time point). For each time point leaves were sampled from the same whorl and same half for each plant and each treatment. Sampling was conducted over a 48 hour timeline (0, 1, 3, 6, 8, 24, 30 and 48 hours after application). The free and conjugated (2-o-ß-D-glucosylsalicylic acid) SA was extracted as described by Verberne *et al.* (2002) with the following modifications. Instead of being ground in liquid nitrogen, fresh leaf samples were ground directly in methanol using a glass tissue macerator. Additionally, the samples were dried under forced air. Dry samples were dissolved in absolute methanol (0.5 ml) and filtered through a 0.45 μm nylon filter (Supelco, Bellefonte, PA). Acetic acid (0.5 ml of 200 mM) was added and the sample was filtered a second time using a 0.45 μm nylon filter. Samples (50 μl) were injected onto a Supercosil LC-18 HPLC column (250 x 4.6mm, Sigma, St. Louis, MO) equipped with a C-18 guard column (7.5 x 4.6 mm, Alltech, Deerfield, IL). Elution was isocratic using 50% methanol containing 200 mM acetic acid with a flowrate of 0.8 ml min^{-1}. Under these conditions, SA had a retention time of approximately 9.6 min at room temperature. Detection was performed using a Model L-4500A diode array detector (Hitachi, Tokyo, Japan). Integration of the salicylic acid peak was performed at 240 nm. External standards were used to generate a standard curve (0.025-2.5 μg ml^{-1}) and determine the SA detection limit of the HPLC. Standards were prepared by dissolving SA in 50% methanol containing 200 mM acetic acid. The SA solutions were filtered through a 0.45 μM syringe filter, then injected (50 μl) and run under the isocratic conditions described above. Salicylic acid concentration following each treatment was analyzed in three separate experiments. However, PBZ treatment was analyzed twice. The experiments were analyzed as a randomized complete block design with repeated measures over time using the Bonferroni Procedure ($P > 0.05$) to determine if the means of SA on a per weight basis differed significantly within a treatment between any time points (SAS, Cary, NC).

To determine the amount of salicylic acid lost during extraction, several untreated leaf samples (2/SA concentration) were amended with exogenous SA (0, 1, 2, 5 and 10 μg). SA, dissolved in 100% methanol, was added to the leaf sample during homogenization, the first step in the extraction procedure. SA was pre-dissolved prior to amending leaf samples in order to ensure it was completely solubilized as a means of reducing variability. The percent of recoverable SA was determined by comparing the integration values obtained by HPLC to a standard curve. Both experiments were repeated twice.

NPR1 Analysis. To examine activation of NPR1, total protein was extracted from sugarbeet leaf tissue at 2 days post treatment with ASM, BmJ, killed BmJ, BMH5E-33, SA or water using a plant fractionated protein extraction kit (Sigma, St. Louis, MO) according to the manufacturer's recommendations. Additionally, total protein was extracted from live BmJ-treated tissue over 48 hours (0, 0.5, 3, 6, 8, 24, and 48 hours after application). Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA) in comparison with bovine serum albumin standards (0-20 μM). Proteins (100 μg/sample) were heated to 60°C for 10 min in sample loading buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol and 0.4% bromphenol blue). When the samples were to be reduced, 50 mM dithiothreitol (DTT) was added to the sample loading buffer. Proteins were resolved on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Both sets of experiments were replicated three times in independent tests. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4°C in primary anti-*Arabidopsis* NPR1 antibody (Provided by Dr. Xinnian Dong, Duke University, Durham, NC; diluted 1:15,000). This was followed by incubation in peroxidase-conjugated goat-anti-rabbit secondary antibody (BioRad, diluted 1:10,000) for 1 hour at room temperature. Colorimetric detection was performed using the 3-amino-9-ethylcarbazole (AEC) staining kit (Sigma, St. Louis, MO).

Since the western blotting procedure used an antibody generated for NPR1 from *Arabidopsis*, the identity of the NPR1 monomer identified in sugarbeet was determined using MALDI-TOF mass spectrometry. Additionally, a corresponding protein detected in an *Arabidopsis* extract through western blotting was also analyzed by MALDI-TOF mass spectrometry. Protein bands from sugarbeet and *Arabidopsis* corresponding to the size of NPR1 identified using the anti-*Arabidopsis* NPR1 antibody, were excised from complementary coomassie blue stained gels run under the identical conditions described above. The

proteins were digested with trypsin using a ProteoProfile trypsin digest kit (Sigma-Aldrich, St. Louis, MO). The resultant peptides were purified and concentrated using C18 zip tips (Millipore, Brillerica, MA). Protein identification was done using a high resolution Bruker Ultraflex MALDI-TOF/TOF Mass Spectrometer with application of MASCOT database search software with manual validation to confirm the highest probability result with two or more peptide MS spectra in conjunction with BLAST searches to suggest homologous proteins from similar organisms. Alignment of the BLAST results for each protein was used to further confirm the protein identity.

RESULTS

Salicylic Acid Production in Sugarbeet. ASM activates resistance downstream of SA and did not lead to an increase in SA over time. The bacterial treatments, both inducers and non-inducers, did not lead to an increase in SA concentration over time. Probenazole led to a slight, transient increase in SA (Table 1).

HPLC and Extraction Limits. It was important to determine the SA lost during the extraction procedure and the range of salicylic acid concentrations reliably detected by HPLC under these experimental conditions. A linear integration range using HPLC was achieved between 0.025 and 2.5 μg of salicylic acid. In order to ensure that SA was not being lost during the extraction procedure, exogenous SA was added to each sample during the first step in the extraction procedure (tissue homogenization). Recovery of SA from leaf extracts amended with exogenous SA prior to sample extraction ranged from 57 to 140 percent (data not shown).

NPR1 Activation. Only the inducers of resistance, live BmJ, SA and ASM, activated NPR1 by 48 hours (Fig. 2). The non-inducer controls, water, dead BmJ and BMH5E-33, did not reduce the NPR1 oligomer. However, the NPR1 monomer could be chemically released through addition of DTT, a reducing agent, in the loading buffer. Following BmJ treatment, the monomeric form of NPR1 was first detectable at 3 hours after BmJ treatment and remained active for 48 hours (Fig. 3). Since the antibody used for detecting sugarbeet NPR1 was developed for *Arabidopsis*, the sugarbeet protein identified was compared to that from *Arabidopsis* via MALDI-TOF mass spectrometry. The spectral data and amino acid sequence alignment confirmed the protein products

Table 1. Salicylic acid in sugarbeet leaf tissue in response to application of systemic resistance inducers.

	Salicylic acid (μg g^{-1} fresh weight)				
Time Post Treatment (h)	**Water**	**ASM**[†]	**BmJ**[†]	**BMH5E-33**[‡]	**PBZ**[†§]
0	1.163	1.296	0.852	0.850	0.896a
1	1.137	1.138	1.025	0.451	1.826ab
3	0.766	1.292	0.808	0.38	2.184b
6	0.651	0.985	0.599	0.275	3.107c
8	1.009	1.296	0.41	0.624	2.753bc
24	0.795	1.065	0.949	0.584	2.105b
30	0.764	0.739	0.545	0.743	0.782a
48	1.149	1.267	0.628	0.356	1.125a

[†] ASM, Acibenzolar-S-methyl; BmJ, *Bacillus mycoides* isolate Bac J; PBZ, Probenazole, inducers of systemic resistance.

[‡] BMH5E-33, *Bacillus pumulis* isolate BMH5E-33, a non-inducer of systemic resistance.

[§] No statistical differences in salicylic acid concentrations within each treatment for water, ASM, BmJ and BMH5E-33. Values as a result of PBZ treatment that are followed by the same letter are not statistically different. Statistical differences were determined using the Bonferroni method (P = 0.05).

detected in both plants was NPR1 based on sugarbeet with 2 peptide matches and *Arabidopsis* with 3 peptide matches and a MOWSE score of 65 and 72 (P = 0.05 cutoff value of 55), respectively (Fig. 4). A total of 55% of the amino acids were identical between the homologs. Ninety-eight differences were positive amino acid substitutions, which are different amino acids with similar chemical properties.

DISCUSSION

In previous reports we described the biochemical response of sugarbeet to *Bacillus mycoides* isolate Bac J, an inducer of systemic resistance and control of Cercospora leaf spot in both glasshouse and some field

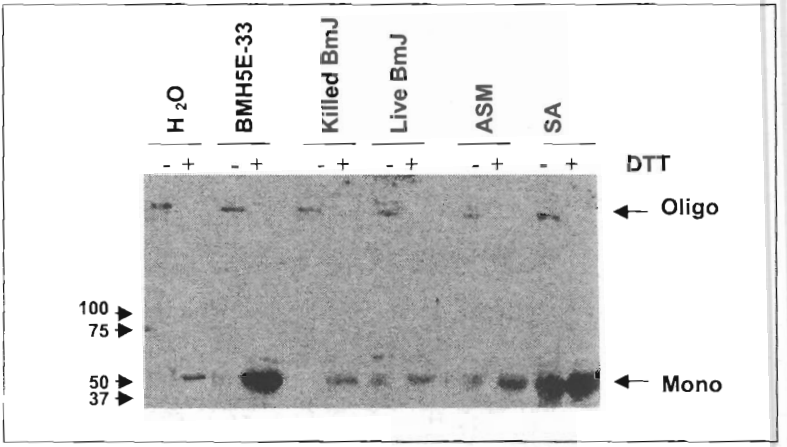

Fig. 2. Detection of NPR1 activation by monomerization. Total protein (100 μg) extracted from sugarbeet leaf tissue 48h after treatment with salicylic acid (SA), acibenzolar-S-methyl (ASM), live *Bacillus mycoides* isolate Bm J (live BmJ), heat-killed *Bacillus mycoides* isolate Bm J (killed BmJ), *Bacillus pumulis* BMH5E-33 and water was separated by SDS-PAGE following heating in sample buffer at 60°C for 10 min in sample buffer with (+) and without (-) dithiothreitol (DTT). The antibody detected the oligomeric (oligo) and monomeric (mono) form of NPR1 in sugarbeet. Numbers represent molecular weight in kilodaltons.

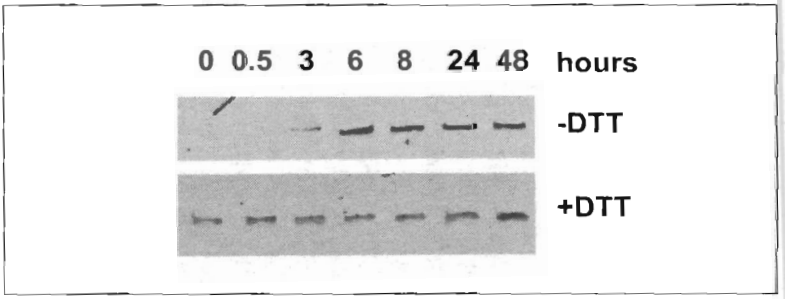

Fig. 3. Activation of NPR1 in sugarbeet leaves following treatment with *Bacillus mycoides* isolate Bm J (BmJ). Total protein (100 μg) extracted at 0, 0.5, 3, 6, 8, 24 and 48 hours post-treatment with BmJ was separated by SDS-PAGE after heating the samples at 60°C for 10 minutes in sample loading buffer with (+) or without (-) dithiothreitol (DTT).

Fig. 4. Amino acid sequence alignment for NPR1 from *Beta vulgaris* (B.v., GenBank accession: AAT57640) and *Arabidopsis thaliana* (A.t., GenBank accession: NP_176610). A total of 55% of the amino acids were identical between the homologs (shown by amino acid designation between the two sequences). Ninety-eight differences were positive amino acid substitutions, which are different amino acids with similar chemical properties (designated with a +). Analysis of the peptide mass fingerprint data for each protein generated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, resulted in a positive identification for NPR1 in sugarbeet (2 peptide matches) and Arabidopsis (3 peptide matches), with a MOWSE score of 65 and 72 (P = 0.05 cutoff value of 55), respectively.

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B.v. 82    KIVVSGDSREVAVHRCVLSSRSSFFRSAFASKREKEKERDKERVVKLELKDLAGDFEVGF 141
           K+V+S D REV+ HRCVLS+RSSFF+SA A+ ++++  +  V   LK++A D+EVGF
A.t. 68    KLVLS-DGREVSFHRCVLSARSSFFKSALAAAKKEKDSNNTAAVKLE-LKEIAKDYEVGF 125

B.v. 142   DSVVAVLGYLYSGKVRNLPRGICVCVDEDCSHEACRPAVDFVVEVLYLSHKFEIVELVSL 201
           DSVV VL Y+YS +VR  P+G+  C DE+C H ACRPAVDF++EVLYL+  F+I EL++L
A.t. 126   DSVVTVLAYVYSSRVRPPPKGVSECADENCCHVACRPAVDFMLEVLYLAFIFKIPELITL 185

B.v. 202   YQRHLLDILDKIAPDDVLVVLSVAEMCGNACDGLLARCIDKIVRSDIDVTTIDKSLPQNV 261
           YQRHLLD++DK+  +D LV+L +A +CG AC  LL RC + IV+S++D+ +++KSLP+ +
A.t. 186   YQRHLLDVVDKVVIEDTLVILKLANICGKACMKLLDRCKEIIVKSNVDMVSLEKSLPEEL 245

B.v. 262   VKQIIDTRKELGFTEPGRVEFPDKHVKRIHRALESDDVELVRMLLKERHTTLDDAYALHY 321
           VK+IID RKELG  P +V+  KHV  +H+AL+SDD+ELV++LLKE HT LDDA ALH+
A.t. 246   VKEIIDRRKELGLEVP-KVK---KHVSNVHKALDSDDIELVKLLLKEDHTNLDDACALHF 301
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B.v. 322  AVAHCDAKTTTELLELGLADVNLRNLRGHTVLHVAAMRKEPKIIVSLLTKGAHPSDITSD 381
          AVA+C+ KT T+LL+L LADVN RN RG+TVLHVAAMRKEP++I+SLL KGA  S+ T +
A.t. 302  AVAYCNVKTATDLLKLDLADVNHRNPRGYTVLHVAAMRKEPQLILSLLEKGASASEATLE 361

B.v. 382  DKKALQIAKRLTKAVDFYKTTEQGKDAPKDRLCIEILEQAERREPLLGEGSVSLAKAGDD 441
          + AL IAK+ T AV+    EQ K + K RLC+EILEQ ++RE +  +  S A A D+
A.t. 362  GRTALMIAKQATMAVECNNIPEQCKHSLKGRLCVEILEQEDKREQIPRDVPPSFAVAADE 421

B.v. 442  LRMKLLYLENRVALARLLFPMEAKVAMDIAQVDGTSEFTLSK----NIADARRNAVDLNE 497
          L+M LL LENRVALA+ LFP EA+ AM+IA++ GT EF ++      +  +R + +
A.t. 422  LKMTLLDLENRVALAQRLFPTEAQAAMEIAEMKGTCEFIVTSLEPDRLTGTKRTSPGVKI 481

B.v. 498  APFILKEEHLQRMKALSKTVELGKRFFPRCSDVLNKIMDAEDLSQLAFLGKDTPEERQRK 557
          APF + EEH  R+KALSKTVELGKRFFPRCS VL++IM+ EDL+QLA   DT E+R +K
A.t. 482  APFRILEEHQSRLKALSKTVELGKRFFPRCSAVLDQIMNCEDLTQLACGEDDTAEKRLQK 541

B.v. 558  RKRYLELQDALTKAFTEDKEE 578
          ++RY+E+Q+ L KAF+ED  E
A.t. 542  KQRYMEIQETLKKAFSEDNLE 562
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experiments. Treatment with BmJ elicits chitinase, ß-glucanase (Bargabus *et al.*, 2002) and hydrogen peroxide (Bargabus *et al.*, 2003) production in sugarbeet, biochemical changes commonly associated with SA-dependent signaling. Additionally, the interaction between SA and H_2O_2 has been implicated in the activation of NPR1, a protein essential for PR-protein gene expression (Mou *et al.*, 2003). Therefore, in the current study, we examined the effect of BmJ treatment on SA levels and NPR1 monomerization in sugarbeet.

Production of SA is transient and free SA is rapidly modified to 2-o-ß-D-glucosylsalicylic acid (Enyedi and Raskin, 1993), a storage compound. Therefore the most accurate measures are from free and conjugated SA together, or total SA, concentrations over time. Total SA remained constant in sugarbeet following BmJ treatment. This was similar to water or BMH5E-33 treatment, both negative controls (Table 1). ASM, a functional analog of SA (Tally *et al.*, 1999), activates the signal transduction cascade downstream of SA, as demonstrated using *nahG Arabidopsis* plants (Chandra-Shekara *et al.*, 2004). Therefore the lack of SA accumulation following ASM application in this study was consistent with other studies. In studies examining SA-dependent resistance, SA levels rise 9-fold over basal levels during resistance activation (Dorey *et al.*, 1997). Our methods detected SA levels as low as 0.025 μg and at a minimum half of all exogenous SA amended to leaf samples was detected. There is a basal level of SA present in all leaf tissue therefore some samples amended with exogenous SA had greater than 100 percent SA recovery. With these standards, a 2-fold or greater increase in endogenous SA would be detected, affirming the lack of SA involvement in BmJ activation of NPR1. Probenazole treatment, an inducer of SA, resulted in increased total SA in sugarbeet (Table 1). This indicates elicitation of SA accumulation does occur in sugarbeet. Since SA accumulation is detectable with other elicitors, this suggests BmJ independence from SA is not an experimental artifact.

NPR1, is constitutively expressed as an inactive multimer. Active monomers are released when NPR1 is reduced, a result of antioxidant compensation for the oxidative burst. Monomerization exposes a nuclear localization signal (Mou *et al.*, 2003) and NPR1 activates transcription of *PR*-genes (Fan and Dong, 2002). In other reports, NPR1 is activated quickly and remains active through 48 hours post treatment (Mou *et al.*, 2003). All inducing treatments, SA, ASM, and live BmJ, activated NPR1 by 48 hours in experiments presented herein. On the other hand, water, dead BmJ and BMH5E-33, non-inducers, did not activate NPR1 (Figure 2). The multimeric state was detected in all samples since it is constitutively expressed. Live BmJ, ASM, and SA treatment lead to

partial reduction of the protein and addition of DTT to the extracts prior to analysis led to full monomerization of NPR1 (Figure 2).

SA-independent activation of NPR1 by BmJ led to the following hypotheses: 1) BmJ activates the SA-dependent signaling cascade downstream of SA or 2) systemic resistance is activated by a novel signaling compound. The former is similar to what is observed with several chemical inducers, such as ASM, 2,6-dichloroisonicotinic acid and N-cyanomethyl-2-chloroisonicotinamide (Yasuda *et al.*, 2003). Other accounts of activation of Bacilli-induced resistance do not reach a congruent conclusion. Ryu *et al.* (2004a and 2004b) have shown two *Bacillus* sp. activate resistance independent of SA. Additionally, the N-terminal region of bacterial flagella is highly conserved (Felix *et al.*, 1999) and plants contain recognition systems to detect this molecular pattern which, in certain instances, elicits NPR1-dependent, SA-independent induction of PR-proteins (Zipfel *et al.*, 2004). However, BmJ does not have flagella. When pathogen-elicited, NPR1 represses jasmonic acid-associated protein production (Spoel *et al.*, 2003). Therefore it is interesting *B. amyloliquefaciens* (strain EXTN-1) induced NPR1-dependent resistance is associated with both SA- and jasmonic acid-dependent defense components (Anh *et al.*, 2002). This demonstrates biocontrol agent activation of NPR1 can have a different outcome than pathogen activation, which may suggest involvement of a novel signaling component. Whether BmJ activates jasmonic acid-associated proteins has not been investigated based on the presumed universal antagonism between SA and JA pathways (Cipollini *et al.*, 2004). The fact that some biocontrol agents concordantly induce normally inhibitory pathways provides additional credence to the idea a unique signal is produced that does not negatively regulate either jasmonic acid or SA associated genes. With continuing contributions, it is becoming apparent that systemic resistance varies depending on the plant and elicitor of resistance. Resistance pathways are not autonomous and cross-communication between the pathways allows for a fine-tuned defensive state that is effective against a particular pathogen. Further investigation may help elucidate whether biocontrol agents activate a different, more diverse subset of defensive components than their pathogenic counterparts. The activation of the NPR1 gene by both BmJ and ASM suggests that BmJ may affect more than just Cercospora leaf spot and may also affect other diseases incited by fungal and bacterial pathogens similar to that observed for ASM. NPR1 acts as a trigger to activate a broad spectrum of antifungal, antibacterial, and antiviral proteins which should establish resistance against more than one specific pathogen. The efficacy of biological control agents is directly tied to effective colonization, which can be impacted

by environmental conditions. Additionally, biocontrol agents are also more effective under lighter disease pressure, therefore biocontrol agent treatment in combination with natural host resistance and reduced rates of chemical pesticides may be the best control option.

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