Systemic Acquired Resistance in Canola Is Linked with Pathogenesis-Related Gene Expression and Requires Salicylic Acid

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ABSTRACT

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Systemic acquired resistance (SAR) is an induced defense response that confers long-lasting protection against a broad range of microbial pathogens. Here we show that treatment of *Brassica napus* plants with the SAR-inducing chemical benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) significantly enhanced resistance against virulent strains of the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* and the fungal pathogen *Leptosphaeria maculans*. Localized preinoculation of plants with an avirulent strain of *P. syringae* pv. *maculicola* also enhanced resistance to these pathogens but was not as effective as BTH treatment. Single applications of either SAR-inducing pretreatment were

effective against *P. syringae* pv. *maculicola*, even when given more than 3 weeks prior to the secondary challenge. The pretreatments also led to the accumulation of pathogenesis-related (PR) genes, including *BnPR-1* and *BnPR-2*, with higher levels of transcripts observed in the BTH-treatment material. *B. napus* plants expressing a bacterial salicylate hydroxylase transgene (*NahG*) that metabolizes salicylic acid to catechol were substantially compromised in SAR and accumulated reduced levels of PR gene transcripts when compared with untransformed controls. Thus, SAR in *B. napus* displays many of the hallmarks of classical SAR including long lasting and broad host range resistance, association with PR gene activation, and a requirement for salicylic acid.

Additional keywords: benzothiadiazole, blackleg, defense mechanisms, signaling.

Systemic acquired resistance (SAR) is an induced disease-resistance state that is achieved in uninfected parts of a plant following localized exposure to pathogens that cause some form of cell death at the site of infection, such as the hypersensitive response (HR) associated with *R*-gene-mediated resistance or disease-induced necrosis (7,28). SAR has been reported in several dicot and monocot species and is effective against a broad range of viruses, bacteria, oomycetes, and fungi (18,30). However, SAR is not effective against all pathogens and the spectrum of resistance varies between plant species (13).

Characteristic features of SAR include the requirement for the phenolic signaling molecule salicylic acid (SA) and an association with the induction of pathogenesis-related (PR) genes and proteins (7). Following exposure to pathogens, SA levels increase substantially at the site of infection (locally) and to a lesser extent, in uninfected (systemic) tissues (28,37). SA accumulation is necessary for SAR, as plants unable to accumulate SA, either through transgenic expression of a bacterial salicylate hydroxylase (*NahG*) gene that metabolizes SA to catechol (5,11,21) or loss-of-function mutations that prevent SA biosynthesis (25), are compromised in SAR. Conversely, exogenous application of SA or its functional analogs benzo (1,2,3) thiadiazole-7-carbothioic acid (S) methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA) lead to enhanced resistance to pathogens (6,9,20).

Increases in SA levels also trigger the local and systemic expression of a subset of PR genes also known as SAR genes (33,

35). The timing of SAR gene expression correlates with the onset and duration of SAR (33,35), and accumulation of SAR gene transcripts is compromised in *NahG* plants (5,21). Although the contribution of individual PR or SAR genes to disease resistance remains unclear (30), their expression provides useful markers for SAR. In particular, *PR-1*, *PR-2*, and *PR-5* have been extensively used as markers for the onset of SAR in *Arabidopsis* and tobacco (7).

Brassica napus is an economically important crop that is grown worldwide and is susceptible to many bacterial and fungal diseases resulting in huge agronomic losses (15). In particular, blackleg disease caused by the ascomycete *Leptosphaeria maculans* is one of the most serious diseases of oilseed *Brassicas*, including canola grade *B. napus* and *B. rapa*. Nevertheless, SAR has yet to be carefully analyzed in oilseed *Brassicas*. Mahuku et al. (23) demonstrated that disease lesions caused by a highly virulent strain of *L. maculans* were reduced significantly when adjacent leaves had been previously infected with a weakly virulent strain. Treatment of *B. napus* with menadione sodium bisulphate (MSB), which appears to induce a form of systemic resistance distinct from SAR, has been shown to be effective against *L. maculans* (1), while application of BTH has been shown to be effective at reducing downy mildew caused by *Peronospora parasitica* in *B. oleracea* (12,17,38) and to provide some control of damping-off caused by *Rhizoctonia solani* in *B. napus* (17). BTH treatment of *B. oleracea* seedlings induced β -1,3 glucanase activity and PR-2 protein accumulation, but had no effect on chitinase activity or the levels of PR-1, PR-3, and PR-5 (38).

The objectives of this study were to assess the effectiveness of SAR against *L. maculans* and to determine whether SAR in *B. napus* is associated with PR gene expression and requires SA. As a reference point, SAR against the bacterial pathogen *Pseudo-*

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monas syringae pv. *maculicola* was also assessed. For both pathogens, protection conferred by preinoculation with a necrogenic pathogen was compared to that induced by treatment with BTH.

MATERIALS AND METHODS

Plant growth conditions. *B. napus* (cv. Westar) plants were grown in the greenhouse at 22°C with 18-h daylight at 190 $\mu\text{E m}^{-2} \text{ s}^{-1}$. Seedlings used for fungal disease testing were grown in cabinets at similar temperature and light conditions with 50% relative humidity.

Production of transgenic *B. napus* expressing the *NahG* gene. *B. napus* (Westar) plants were transformed by *Agrobacterium*-mediated transformation according to Tsang et al. (32). The plasmid used for transformation contained the *NahG* gene driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter described by Liu et al. (22). Rooted plants were transferred to pots for seed and tested for *NahG* gene expression by northern blot analysis. All transgenic *NahG* plants used in this study were from the same transgenic event. To ensure that all individuals analyzed were indeed genetically transformed, a cotyledon from each was tested for the expression of the *Escherichia coli uidA* (*GUS*) gene which was incorporated into the T-DNA (27) and encodes a β -D-glucuronidase.

Chemical treatment. Ten-day-old cotyledons (for fungal disease testing), 4-week-old plants (for bacterial disease testing), or plants at different ages (for time course studies) were sprayed with a freshly made aqueous solution of BTH (37.5 μg of active ingredient [a.i.] ml^{-1} ; 178 μM) (Syngenta Crop Protection Canada, Inc., Guelph) containing 0.01% vol/vol Tween 20. This concentration is similar to that previously found to be effective in *Arabidopsis* (20). Control plants were either sprayed with water containing Tween 20 or received no treatment.

Pathogen inoculations for SAR against *P. syringae* and bacterial quantification. *P. syringae* pv. *maculicola* strains were grown at 30°C in yeast-tryptone (2YT) medium (29) for 16 h, pelleted in a Sorvall RCA5C centrifuge with GSA rotor (Thermo Fisher Scientific Inc., Waltham, MA) at 5,000 rpm for 15 min and resuspended in 10 mM MgCl_2 . Bacterial titer was estimated by optical density at 600 nm, with an OD_{600} of 1 equivalent to 1×10^8 colony forming units (CFU) ml^{-1} , and diluted to the desired concentrations with 10 mM MgCl_2 . Throughout this study, avirulent *P. syringae* pv. *maculicola* 1120B was used at 1×10^6 CFU ml^{-1} while virulent *P. syringae* pv. *maculicola* 1848B was used at 1×10^5 CFU ml^{-1} .

To assess the longevity of SAR, either the cotyledons (for the week 3 time point) or the first and second true leaves (all other time points) were infiltrated with avirulent *P. syringae* pv. *maculicola* 1120B, from the abaxial side, with a 1-ml plastic syringe. Control plants were either infiltrated with 10 mM MgCl_2 alone or received no pretreatment. Secondary inoculations on third and fourth true leaves were done on 4-week-old plants by infiltrating with virulent *P. syringae* pv. *maculicola* 1848B. Three days later, eight leaf discs (4-mm diameter) per plant were collected randomly from third and fourth leaves. Leaf samples were ground in 500 μl of 10 mM MgCl_2 , serially diluted, and spread on *Pseudomonas* Agar-F medium (Difco, Sparks, MD) plates. These were incubated at 30°C for 3 days before recording the number of colonies. Each time point represents nine samples, with each sample consisting of eight leaf discs.

To monitor the growth of virulent *P. syringae* pv. *maculicola* 1848B at different times following its infiltration into third and fourth leaves, samples (leaf discs) were randomly collected from 4-week-old plants daily, from 1 to 5 days following infiltration, and processed as described above. The effect of SAR was assessed by first infiltrating the first and second leaves of the above plants with *P. syringae* pv. *maculicola* 1120B or 10 mM

MgCl_2 , or by spraying entire plants with BTH, 4 days prior to the secondary infiltration with *P. syringae* pv. *maculicola* 1848B.

For comparison of SAR between untransformed and *NahG* plants, secondary inoculations were done 4 days after BTH treatment or primary inoculation with avirulent *P. syringae* pv. *maculicola* 1120B, and growth of virulent *P. syringae* pv. *maculicola* was quantified 3 days following the secondary inoculations.

For all *P. syringae* pv. *maculicola* disease tests, the average number of CFU per leaf disc was analyzed statistically using an unpaired Student's *t* test at $P = 0.05$ (36). A statistically significant reduction in bacterial titer was interpreted as an increase in disease resistance, and accordingly, effective deployment of SAR. All experiments were repeated at least twice.

Pathogen inoculation for SAR against *L. maculans* and disease assessment. *L. maculans* GL-11, containing the *E. coli GUS* gene, was grown in black light (300–400 nm) at 24°C on solid V8-juice agar medium (20% vol/vol V8 juice, 0.75% CaCO_3 , 1.5% agar, 100 $\mu\text{g ml}^{-1}$ streptomycin, 50 $\mu\text{g ml}^{-1}$ hygromycin, and 40 $\mu\text{g ml}^{-1}$ rose Bengal). Pycnidiospore suspensions were prepared according to Mahuku et al. (23) to a final concentration of 1×10^7 spores ml^{-1} in water and stored at -80°C .

Ten-day-old cotyledons were used for testing SAR against *L. maculans*. Primary inoculations were done on one of the cotyledons by infiltrating with avirulent *P. syringae* pv. *maculicola* 1120B on the abaxial side at a concentration of 1×10^6 CFU ml^{-1} . For controls, cotyledons were infiltrated with 10 mM MgCl_2 alone. Secondary inoculations were done on cotyledons from seedlings pretreated with BTH or preinoculated with avirulent *P. syringae* pv. *maculicola* 1120B by placing 10 μl of 1×10^7 spore ml^{-1} *L. maculans* suspension onto a wound in their center made using a pair of Allegiance tissue teeth forceps (VWR Intl., Mississauga, ON, Canada; cat. no. 25601-080). The seedlings were misted for the first 2 days (95% relative humidity) and after 6 more days, they were harvested, frozen in liquid nitrogen, and stored at -80°C .

Histological and fluorometric GUS assays were performed according to Jefferson et al. (16) using 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid cyclohexyl ammonium salt (X-Gluc) and 4-methylumbelliferone β -D-galactopyranoside (MUG) as substrates, respectively. Protein concentration was quantified by the Bradford assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Fluorometric GUS assay results are presented as pmol methylumbelliferone $\mu\text{g protein}^{-1} \text{ min}^{-1}$. Trypan blue staining of *NahG* cotyledons was performed as previously described (22) except that samples were cleared with 70% ethanol. Sections were mounted on a slide and photographed using an Optronics International DEI 750 digital microscope camera (Chelmsford, MA). All experiments were repeated at least twice.

Salicylic acid analysis (adapted from Verberne et al. [34]). Leaf material (≈ 300 mg) was ground in liquid nitrogen and placed into a 15-ml round bottom glass culture tube containing 3 ml of extraction solvent (90% methanol, 9% water, 1% glacial acetic acid) and 200 ng of internal standard (3, 4, 5, 6-tetradeuterio-salicylic acid, CDN Isotopes Inc., Pointe-Claire, Québec).

After sonication for 5 min at 30°C in a sonicating water bath, the material was centrifuged for 5 min at 2,000 rpm at 10°C. The supernatant was removed and the pellet extracted with another 3 ml of extraction solvent. After centrifugation the pellet was extracted again, this time with 2 ml of methanol. The supernatants were pooled and then the methanol was evaporated under a stream of nitrogen. The remaining aqueous solution was placed on ice, and its pH adjusted to 10 with 0.1N NaOH. The mixture was then extracted with 3 ml of dichloromethane. The aqueous layer was transferred to a new tube and the dichloromethane layer was back-extracted with 2 ml of 0.1N NaOH. The pooled aqueous layers were acidified with 5% HCl and extracted 3 times with a 1-ml mixture of ethylacetate:cyclohexane (1:1, vol/vol).

The solvents were removed under a stream of nitrogen and the residue was dissolved in 10 μ l of derivitizing reagent (1:1 mixture of N,O-bis(trimethylsilyl) acetamide and pyridine).

Gas chromatography/mass spectrometry (GC/MS) analysis was accomplished using an Agilent 6890 GC equipped with an auto injector split 30:1 onto a DB-5MS column (30M $\times$ 0.25 mm i.d., J&W Scientific, Folsom, CA) that was temperature-programmed to ramp from 175°C to 300°C at 5°C min⁻¹ (8). The column was connected to an Agilent 5973 mass selective detector. For maximum sensitivity the detector was run under standard conditions with a limited scan range of 265 to 275 m/z that gave 37.9 scans per second. For maximum selectivity and accurate quantitation, ions specific to the compounds of interest were monitored and integrated. For the trimethylsilyl derivative of SA 267 m/z was used, and for the tetradeutero internal standard, 271 m/z was used. The detection limit of the TMS derivative under these conditions was $\approx$ 0.1 ng μ l⁻¹ and the responses were linear within the concentration range used.

Northern blot analysis. Methods for total RNA isolation and northern analysis were as described by Liu et al. (22). Total RNA from *B. napus* plants was isolated from frozen samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad). Five micrograms of RNA was denatured and separated on a 1.2% (wt/vol) agarose 3% (vol/vol) formaldehyde gel, photographed under UV light to record amounts loaded, and blotted to nylon membrane (GeneScreen Plus Hybridization Transfer Membrane; NEN Life Sciences Products, Inc., Boston). Membranes were cross-linked with UV light, hybridized at 65°C in QuickHyb solution (Stratagene, La Jolla, CA) with probes radiolabelled with (α -³²P)dCTP using random primers (Invitrogen, Burlington, Ontario). Two hours after hybridization, membranes were washed twice at room temperature in 2 $\times$ SSC 1% SDS for 15 min; once in 0.1 $\times$ SSC and 0.1% SDS at 65°C for 30 min; and later exposed onto X ray film (Sterlin Diagnostics, Newark, DE) at -80°C. For rehybridization with different gene probes, membranes were stripped by boiling for 30 min in 0.1 $\times$ SSC and 0.1% SDS. Hybridization probes contained the entire coding regions of *Brassica PR-1* (U70666, herein referred to as *BnPR-1*) and *PR-2* (X77990, *BnPR-2*) genes previously shown to be induced following pathogen challenge (10,26) and BTH (14). Because we were not aware of any

characterized *Brassica PR-5*-related genes expressed under these conditions, we chose a gene originally isolated from flower buds for analysis, U71244 (4). Coding regions were isolated by reverse transcriptase polymerase chain reaction amplification of mRNA isolated from *B. napus* seedlings treated with 2 mM SA (Sigma, St. Louis, MO) for 18 h using the following oligonucleotide primers: *BnPR-1* (5'-ATGAAAGTCACTAACTGTTCTCGAC-3' and 5'-GCCAGTAACTAGGTAACGGATAA-3'); *BnPR-2* (5'-GTGTTAGCATCATCACCAGTGTGCTG-3' and 5'-GGAGATTAGTTAACTTAACACCATATTTAAGCTG-3'); *BnPR-5* (5'-CAATGGCTTCACGAAACCTCTTCAACTTCG-3' and 5'-GTGATTTTAACGGCGATGGTGAGGGCAAAA-3'). Isolated fragments were ligated into pTOPO4 cloning vector (Stratagene) and verified by sequencing.

RESULTS

SAR is effective at reducing disease caused by *P. syringae* pv. *maculicola*. In *Arabidopsis*, SAR against *P. syringae* is very effective and has been characterized extensively (2,3,33). Accordingly, we initiated our studies of SAR in *B. napus* using this bacterial pathogen. As a first step, a collection of *P. syringae* pv. *maculicola* strains were infiltrated into leaves of *B. napus* (cv. Westar) to identify those capable of inducing disease symptoms, which could be used to monitor the effectiveness of SAR treatments. At the same time, strains were assessed for their capacity to elicit an HR, and thus having the potential to trigger SAR. One representative strain capable of inducing disease symptoms (*P. syringae* pv. *maculicola* 1848B) and one capable of eliciting an HR response (*P. syringae* pv. *maculicola* 1120B) were chosen for further study.

The effectiveness of preinoculation with an avirulent (necrogenic) pathogen at reducing disease was tested by infiltrating the first and second true leaves of plants with 1×10^6 CFU ml⁻¹ of *P. syringae* pv. *maculicola* 1120B and subsequently infiltrating the third and fourth leaves with 1×10^5 CFU ml⁻¹ of virulent *P. syringae* pv. *maculicola* 1848B. Leaves infiltrated with avirulent *P. syringae* pv. *maculicola* 1120B are herein referred to as local leaves, while the third and fourth leaves from these plants are referred to as systemic leaves. To assess the effectiveness of BTH at reducing disease, a separate group of plants was sprayed with 37.5 μ g a.i. ml⁻¹ BTH prior to infiltration with *P. syringae* pv. *maculicola* 1848B. These plants were grown in parallel and under the same conditions as those infiltrated with avirulent *P. syringae* pv. *maculicola* 1120B, and BTH treatments were scheduled to coincide with preinoculation treatments. Disease was assessed by visually monitoring symptoms and quantifying viable bacteria in the third and fourth leaves. Preliminary experiments indicated that preinoculation with 10 mM MgCl₂ or spraying with a Tween 20 solution had no significant effect on growth of *P. syringae* pv. *maculicola* 1848B (data not shown). Accordingly, control plants in most experiments did not receive any pretreatments. Preliminary trials suggested that reduction of disease symptoms was greatest when *P. syringae* pv. *maculicola* 1848B was inoculated 4 days after primary treatments. Therefore, these conditions were selected to quantify bacterial growth at different times following infiltration with *P. syringae* pv. *maculicola* 1848B.

In leaves from control plants, bacteria multiplied to 4×10^4 CFU per leaf disc after one day and increased to 1.6×10^6 CFU per leaf disc after 4 days before tapering off (Fig. 1). Leaves from plants that had been preinoculated with the avirulent pathogen displayed a 4.1- (day 2 and day 5) to 31-fold (day 4) reduction in the titer of virulent *P. syringae* pv. *maculicola* 1848B, compared to control plants that received no pretreatments sampled at the same time. Leaves from plants treated with BTH showed a dramatic reduction of *P. syringae* pv. *maculicola* 1848B growth (75- to 1,328-fold) when compared to control plants. Statistical analysis using

Fig. 1. In planta quantification of the growth of the virulent strain of *Pseudomonas syringae* pv. *maculicola* 1848B in *Brassica napus* leaves at different times following systemic acquired resistance (SAR)-inducing pretreatments. Control plants did not receive any pretreatment. SAR treatments consisted of preinoculation with avirulent *P. syringae* pv. *maculicola* 1120B (1×10^6 CFU ml⁻¹) or spraying plants with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) (37.5 μ g active ingredient ml⁻¹). Secondary inoculations with the virulent *P. syringae* pv. *maculicola* strain 1848B (1×10^5 CFU ml⁻¹) were performed 4 days after primary treatments. Bacterial counts (CFU per leaf disc) were determined for each treatment at the indicated times after secondary inoculation. Each sample consisted of eight leaf discs and every data point represents the mean $\pm$ SE of nine samples. At every time point, values for the *P. syringae* pv. *maculicola* 1120B and BTH treatments are statistically different from the corresponding values from the control (no SAR) leaves ($P \leq 0.05$, Student's *t* test).

Student's *t* test at $P \leq 0.05$ indicated that, in both the SAR treatments, bacterial titers measured at every time point were significantly lower than those from control leaves at the corresponding time. Extracts from leaves infiltrated with buffer alone did not yield any colonies at the dilutions used for the infected plants. Preinoculation with the avirulent pathogen resulted in a marked reduction of visible disease symptoms in leaves subsequently infiltrated with virulent bacteria, while plants treated with BTH displayed no disease symptoms following infiltration with the virulent bacteria (data not shown).

SAR against *P. syringae* pv. *maculicola* is long lasting. To determine the longevity of SAR, bacterial titers were quantified in systemic leaves infected with virulent *P. syringae* pv. *maculicola* 1848B at various times after pretreatment with avirulent *P. syringae* pv. *maculicola* 1120B or BTH. In order to make comparisons between treatments more meaningful, the secondary inoculations were all performed at the same time, on plants of the same age, with the pretreatments having been performed at different times. As shown in Figure 2A, preinoculation with avirulent *P. syringae* pv. *maculicola* 1120B resulted in a 14- to 19-fold reduction in bacterial growth. Values from all of the time points were statistically different from those obtained in the control plants ($P \leq 0.05$), indicating that preinoculation with avirulent *P. syringae* pv. *maculicola* 1120B can effectively increase disease resistance even when the secondary inoculation was performed 3 weeks after the pretreatment, the longest time difference tested in this study.

Treatment with BTH resulted in a 95- to 1,077-fold reduction in bacterial growth, with the maximum reduction observed when the secondary inoculation was performed 4 to 7 days after the primary treatment (Fig. 2A). All values measured in leaves treated with BTH were statistically lower than the control ($P \leq 0.05$). These results indicate that both SAR treatments tested provide long lasting protection against virulent *P. syringae* pv. *maculicola* in *B. napus* and confirm the data presented in Figure 1 that treatment with BTH is more effective than preinoculation with the avirulent pathogen *P. syringae* pv. *maculicola* 1120B under the experimental conditions used.

SAR against *P. syringae* pv. *maculicola* is correlated with the accumulation of PR gene transcripts. Changes in PR gene expression associated with SAR induction were analyzed by northern blot hybridization in leaf samples collected at various times following pretreatments (Fig. 2B). In local leaves inoculated with avirulent *P. syringae* pv. *maculicola* 1120B, high levels of *BnPR-1* and *BnPR-2* mRNA were already detected 1 day postinfection (Fig. 2B), the shortest time interval used between primary and secondary inoculations in this study. Sampling of earlier time intervals detected very low levels of *BnPR-1* in local leaves after 1 and 4 h, and high levels by 8 h (Fig. 2C). Steady-state levels of PR gene mRNA increased slightly during the first week before starting to decline at day 14 (Fig. 2B). In systemic leaves, levels of the above gene transcripts were initially substantially lower than observed in the local leaves and gradually increased until day 7 (Fig. 2B and C). Levels then remained relatively constant and similar to those detected in infected leaves for the remainder of the experiment. Treatment with BTH induced *BnPR-1* and *BnPR-2* transcript accumulation to levels comparable to the highest levels observed in local leaves (Fig. 2B). Expression of *BnPR-5* was not detected in any of the tissues analyzed, while transcripts from none of the PR genes analyzed were detected in leaves from untreated plants at any of the developmental stages considered (Fig. 2B).

PR transcripts were also monitored in leaves at different times following infection with the virulent *P. syringae* pv. *maculicola* strain 1848B (Fig. 2D). In plants that had not received SAR pretreatments, infection with *P. syringae* pv. *maculicola* 1848B was sufficient to induce *BnPR-1* and *BnPR-2* expression as early as 1 day postinfection. In plants where first and second leaves had

Fig. 2. Systemic acquired resistance (SAR) response and *BnPR* gene transcript accumulation in wild-type *Brassica napus* plants, induced by *Pseudomonas syringae* pv. *maculicola* 1120B or benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). **A**, Plants were either preinoculated with a 1×10^6 CFU ml⁻¹ suspension of avirulent *P. syringae* pv. *maculicola* 1120B or sprayed with 37.5 μ g active ingredient ml⁻¹ BTH for the amount of time indicated before a secondary challenge inoculation with the virulent *P. syringae* pv. *maculicola* strain 1848B (1×10^5 CFU ml⁻¹). Control plants did not receive any pretreatments. Three days later, eight leaf discs per sample were collected and bacterial titers quantified. Each data point represents the mean $\pm$ SE of nine samples. At every time point, values for preinoculation and BTH treatments are statistically significant from the control ($P \leq 0.05$, Student's *t* test). **B**, Northern blot analyses of *BnPR-1*, *BnPR-2*, and *BnPR-5* transcript accumulation at different times (in days) following the SAR-inducing pretreatments described in A. L = local, infected leaf; S = systemic, noninfected leaf. **C**, Northern blot analyses of *BnPR-1* transcript accumulation in local leaves at early stages following challenge inoculation with the avirulent *P. syringae* pv. *maculicola* strain 1120B (1×10^6 CFU ml⁻¹). **D**, Northern blot analyses of *BnPR-1* and *BnPR-2* accumulation in systemic leaves at different times (in days) following challenge inoculation with the virulent *P. syringae* pv. *maculicola* strain 1848B (1×10^5 CFU ml⁻¹). Plants had received SAR-inducing pretreatments 4 days previously, as described in A. **B**, **C**, and **D**, Five micrograms of total RNA was loaded for each sample and hybridized with radioactive labeled DNA probes. The same filters were used for *BnPR-1*, *BnPR-2*, and *BnPR-5*. These were stripped of hybridizing DNA between each round of hybridization. Photos of ethidium bromide-stained gels are included as a measure of RNA loading.

been inoculated 4 days previously with avirulent *P. syringae* pv. *maculicola* 1120B, systemic leaves infected with *P. syringae* pv. *maculicola* 1848B expressed slightly higher levels of *BnPR-1* transcripts than observed in plants that did not receive the pre-treatment, but appeared to have similar levels of *BnPR-2*, except at day 0, when *BnPR-1* and *BnPR-2* transcripts were detected only in systemic leaves from plants previously inoculated with the avirulent bacterium. Transcript levels of both of these PR genes were higher in BTH-treated plants than those receiving no pre-treatment or preinoculated with *P. syringae* pv. *maculicola* 1120B. Expression of *BnPR-5* was not detected in any of the leaves infected with *P. syringae* pv. *maculicola* 1848B (data not shown). Together, these results indicate that SAR against *P. syringae* pv. *maculicola* in mature *B. napus* plants is associated

with the induction of PR genes at both the induction and manifestation stages, and that there is a positive relationship between the effectiveness of SAR and the steady-state level of PR transcripts.

SAR is effective against *L. maculans*. Studies have shown that early infections of *L. maculans* cause the greatest damage, with infections before the six-leaf stage causing severe yield losses (15). Stems are most susceptible to infection when the plants are in cotyledon or one- to two-leaf stage. Therefore, we tested the effectiveness of SAR against *L. maculans* in *B. napus* plants at the cotyledon stage.

To trigger SAR, either one cotyledon from 10-day-old seedlings was infiltrated with avirulent *P. syringae* pv. *maculicola* 1120B (1×10^6 CFU ml⁻¹) or the seedling was sprayed with 37.5 µg a.i. ml⁻¹ BTH. Four days later, two 10-µl drops of *L. maculans* spore suspension (1×10^7 ml⁻¹) were placed on the cotyledons. In cases where one cotyledon had been preinoculated with avirulent *P. syringae* pv. *maculicola* 1120B, the secondary infection with *L. maculans* was performed on the other cotyledon. The *L. maculans* strain used in this study (GL-11) expressed the *GUS* gene. Accordingly, qualitative and quantitative analysis of GUS activity was exploited as a measure of fungal growth.

Histological staining for GUS activity 8 and 13 days after *L. maculans* infection revealed that preinoculation of one of the cotyledons with avirulent *P. syringae* pv. *maculicola* 1120B substantially decreased the area of fungal growth (Fig. 3A). This reduction was even more dramatic in cotyledons from seedlings treated with BTH. Quantitatively, GUS activity was reduced by 2.3-fold in seedlings preinoculated with avirulent *P. syringae* pv. *maculicola* 1120B and 77-fold following treatment with BTH (Fig. 3B). These differences were statistically significant ($P \leq 0.05$) and indicate that SAR induced at the cotyledon stage of *B. napus* plants is effective against *L. maculans*.

BnPR-1 and *BnPR-2* transcripts accumulated at high levels in cotyledons infected with avirulent *P. syringae* pv. *maculicola* 1120B and following BTH treatment (Fig. 4A). Expression of PR genes was much lower in the systemic cotyledons that were not infected with *L. maculans*. In the absence of pretreatment, infection with *L. maculans* resulted in the accumulation of low levels of *BnPR-1* and *BnPR-2* mRNA (Fig. 4B). Preinoculation with avirulent *P. syringae* pv. *maculicola* 1120B resulted in higher levels of PR gene transcripts in cotyledons infected with *L. maculans*, while spraying with BTH induced the highest levels of PR gene mRNA in these tissues. Thus, as observed in mature plants infected with virulent *P. syringae* pv. *maculicola* 1848B, there is a positive relationship between the effectiveness of SAR against *L. maculans* and the expression of PR genes in *B. napus* seedlings.

Systemic acquired resistance is compromised in *NahG* *B. napus* plants. The requirement of SA for SAR in *B. napus* was tested in transgenic plants expressing the bacterial salicylate hydroxylase gene (*NahG*; Fig. 5A) that converts SA to catechol (11). To confirm the efficacy of the *NahG* transgene, levels of free SA were measured in local and systemic leaves of plants 4 days after preinoculation with avirulent *P. syringae* pv. *maculicola* 1120B. As shown in Figure 5B, inoculation of untransformed (wild-type) plants with avirulent *P. syringae* pv. *maculicola* 1120B resulted in a 28-fold increase in SA levels in local tissues and a roughly twofold increase in systemic leaves. In contrast, the *NahG* plants did not show any substantial increase in free SA levels in either locally infected or systemic leaves (Fig. 5B).

In the absence of SAR inductive pretreatments, bacterial titers measured in leaves of *NahG* plants infected with virulent *P. syringae* pv. *maculicola* 1848B were twofold higher than those measured in untransformed plants (Fig. 5C). This difference was statistically significant ($P \leq 0.05$). To determine if SAR could be induced in *NahG* plants, the third and fourth leaves of untransformed and *NahG* plants were infiltrated with the virulent

Fig. 3. Effectiveness of systemic acquired resistance-inducing pretreatments in wild-type *Brassica napus* cotyledons against *Leptosphaeria maculans*. Six-day-old *B. napus* seedlings were either sprayed with water (control), preinoculated with 1×10^6 CFU ml⁻¹ of avirulent *Pseudomonas syringae* pv. *maculicola* 1120B, or sprayed with 37.5 µg active ingredient ml⁻¹ benzo-(1,2,3)-thiadiazole-7-carboxylic acid S-methyl ester (BTH). Four days later, 10-µl drops of *L. maculans* spores (1×10^7 CFU ml⁻¹) were placed on cotyledons and incubated for 8 or 13 days. Different cotyledons from the same seedlings received the primary and secondary inoculations. **A**, Histological staining of *L. maculans* fungal hyphae for *Escherichia coli* β-D-glucuronidase (*GUS*) gene expression using X-Gluc. Hyphal growth 8 or 13 dpi (days post inoculation) of the fungus is indicated by blue coloration; **B**, Quantitative GUS expression analyses in 8-dpi cotyledons using 4-methylumbelliferone β-D-galactopyranoside (MUG) as substrate. Reduction in growth of *L. maculans* is indicated by decreased GUS expression. Each bar represents the mean ± SE of six samples. GUS activity measured in cotyledons from seedling preinoculated with *P. syringae* pv. *maculicola* 1120B or sprayed with BTH were statistically lower than control values ($P \leq 0.05$, Student's *t* test).

P. syringae pv. *maculicola* 1848B 4 days after preinoculation of first and second leaves with the avirulent *P. syringae* pv. *maculicola* 1120B, and bacterial growth quantified as described above. Untransformed plants showed a fourfold reduction in the growth of *P. syringae* pv. *maculicola* 1848B whereas the *NahG* plants did not show any significant decrease (Fig. 5C).

In *Arabidopsis* and tobacco, loss of SAR observed in *NahG* plants can be rescued by treatment with BTH (9,20). This chemical is thought to act at the same point as, or downstream of, SA in the signaling pathway leading to SAR (9,20). To test whether the loss of resistance in *NahG B. napus* was specifically attributed to interference with SA signaling, plants were treated with BTH and assessed for disease resistance. Following BTH treatment, titers of virulent *P. syringae* pv. *maculicola* 1848B in leaves of *NahG* plants were significantly lower (77-fold) than those measured in the absence of pretreatment or following preinoculation with avirulent *P. syringae* 1120B (Fig. 5C). These differences were statistically significant ($P \leq 0.05$). However, titers in *NahG* plants treated with BTH continued to be higher than those measured in leaves of untransformed plants treated with BTH.

When compared with untransformed plants, both infected (local) and systemic leaves of *NahG* plants displayed substantial

Fig. 4. *BnPR* transcript accumulation in wild-type *Brassica napus* cotyledons following systemic acquired resistance-inducing pretreatments. Five micrograms of total RNA was loaded for each sample and hybridized with radioactive labeled DNA probes. The same filters were used for *BnPR-1* and *BnPR-2* and stripped of hybridizing DNA between each round of hybridization. Photos of ethidium bromide-stained gels are included as a measure of RNA loading. **A**, Northern blot analyses of *BnPR-1* and *BnPR-2* at different times following pretreatments. *P. syringae* pv. *maculicola* 1120B indicates preinoculation with avirulent *Pseudomonas syringae* pv. *maculicola* 1120B (1×10^6 CFU ml⁻¹); BTH indicates seedlings were sprayed with 37.5 µg active ingredient ml⁻¹ benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester. L = local cotyledon and S = systemic cotyledon. **B**, Northern blot analyses of *BnPR-1* and *BnPR-2* at different times in cotyledons following challenge inoculation with *Leptosphaeria maculans* (1×10^7 CFU ml⁻¹) spores. Cotyledons had previously received the pretreatments for 4 days as described in **A**. Separate cotyledons of the same seedling were used for seedlings that received both bacterial and fungal inoculations.

Fig. 5. Systemic acquired resistance (SAR) response against *Pseudomonas syringae* and *BnPR* gene expression in *NahG Brassica napus* plants. **A**, Northern blot showing expression of the *NahG* gene in transgenic *B. napus* plants. Five micrograms of total RNA was loaded for each sample and probed with radioactive labeled *NahG* probe. A photo of the ethidium bromide-stained gel is included as a measure of RNA loading; **B**, Gas chromatography/mass spectrometry (GC/MS) quantification of free salicylic acid in leaf tissues four days after infiltration with 1×10^6 CFU ml⁻¹ of avirulent *Pseudomonas syringae* pv. *maculicola* 1120B. L = local leaf; S = uninfected, systemic leaves from plants infiltrated with the avirulent bacteria; and C = untreated control leaves. Each bar represents the mean $\pm$ SE of three samples. **C**, SAR response in wild-type and *NahG* plants. Three-week-old wild-type (open bars) or *NahG* transgenic (hatched bars) *B. napus* plants were infiltrated with 10 mM MgCl₂ (control), preinoculated (first and second leaves) with 1×10^6 CFU ml⁻¹ of avirulent *P. syringae* pv. *maculicola* 1120B or sprayed with 37.5 µg active ingredient ml⁻¹ benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). Four days later, the third and fourth leaves of each plant were challenge-inoculated with the virulent *P. syringae* pv. *maculicola* strain 1848B. Three days after the second inoculation, eight leaf discs were collected randomly from third and fourth leaves and bacterial titers were quantified. Each data point represents the mean $\pm$ SE of nine samples. All treatments for wild-type and *NahG* are statistically different from the wild-type MgCl₂ control ($P \leq 0.05$, Student's *t* test). Values for *NahG* plants preinoculated with *P. syringae* pv. *maculicola* 1120B or sprayed with BTH are also statistically significant from the *NahG* MgCl₂ control; **D**, Northern blot analyses show *BnPR-1* transcript accumulation in wild-type and *NahG B. napus* plants 0, 1, and 4 days after SAR pretreatment. C = untreated control; L = local (first and second) leaf inoculated with 1×10^6 CFU ml⁻¹ avirulent *P. syringae* pv. *maculicola* 1120B; S = systemic (third and fourth) leaves of the same plants; and B = leaf sprayed with 37.5 µg active ingredient ml⁻¹ BTH. Five micrograms of total RNA was loaded for each sample and hybridized with radioactive labeled DNA probes. A photo of the ethidium bromide-stained gel is included as a measure of RNA loading.

reductions of steady-state *BnPR-1* transcripts 1 day following challenge with avirulent *P. syringae* pv. *maculicola* 1120B (Fig. 5D). Four days following infection, levels of *BnPR-1* mRNA in *NahG* plants were similar to those observed in untransformed plants. Treatment of *NahG* plants with BTH also resulted in the accumulation of relatively high levels of *BnPR-1* transcripts (Fig. 5D).

The binary T-DNA plasmid used to generate the *NahG* plants possessed a chimeric selectable marker gene containing *GUS* (27). Accordingly, we were unable to use this reporter to measure growth of *L. maculans*. Instead, fungal growth was assessed by monitoring mycelial mass using trypan blue staining. Regardless of whether *NahG* seedlings were preinoculated with avirulent *P. syringae* pv. *maculicola* 1120B, the fungus grew to entirely cover the cotyledons (Fig. 6A, bottom row). By comparison, fungal growth was not as extensive on wild-type cotyledons and preinoculation was modestly effective at reducing fungal growth (Fig. 6A, top row). Trypan blue staining of *NahG* cotyledons after BTH treatment indicated that growth of *L. maculans* was significantly reduced when compared with cotyledons that did not receive any pretreatment (Fig. 6A).

When compared with untransformed cotyledons, levels of *BnPR-1* transcripts were substantially lower in local and systemic cotyledons of *NahG* seedlings 8 h after inoculation with avirulent *P. syringae* pv. *maculicola* 1120B (Fig. 6B). Levels remained lower in both local and systemic *NahG* cotyledons 1 and 2 days after inoculation, although the differences between untransformed cotyledons were not as pronounced. Therefore, the loss of SAR observed in the *NahG* seedlings, as observed in mature plants (Fig. 5D), is associated with reduced accumulation of *BnPR-1* transcript, particularly during the early stages following preinoculation with the avirulent pathogen. Altogether, the analysis of *NahG* transgenics indicate that the SA signaling pathway is critical for effective SAR against *P. syringae* and *L. maculans* in *B. napus*.

DISCUSSION

We have demonstrated that localized preinoculation with an avirulent pathogen and application of the chemical BTH effectively enhances resistance of *B. napus* plants to virulent strains of

Fig. 6. Systemic acquired resistance (SAR) response against *Leptosphaeria maculans* and *BnPR* gene expression in *NahG* *B. napus* cotyledons. **A**, Trypan blue staining of cotyledons from wild-type and *NahG* seedlings 13 days after infection with *L. maculans*. Control = no pretreatment; *P. syringae* pv. *maculicola* 1120B = the other cotyledon of the seedling, infiltrated with 1×10^6 CFU ml^{-1} of avirulent *P. syringae* pv. *maculicola* 1120B 4 days before infection with *L. maculans*; and BTH = seedlings sprayed with $37.5 \mu\text{g}$ active ingredient ml^{-1} benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) 4 days before infection with *L. maculans*. Hyphal growth is indicated by blue coloration; **B**, Northern blot analyses of *BnPR-1* transcript accumulation in wild-type and *NahG* *B. napus* cotyledons at different times following pretreatment with 1×10^6 CFU ml^{-1} of avirulent *P. syringae* pv. *maculicola* 1120B. L = local cotyledon and S = systemic cotyledon. A photo of an ethidium bromide-stained gel is included as a measure of RNA loading.

the bacterial pathogen *P. syringae* pv. *maculicola* and the fungal pathogen *L. maculans*. Importantly, we show that enhanced resistance to both pathogens displays the hallmarks of a typical SAR response, including the accumulation of PR gene transcripts and a requirement for SA.

Preinoculation with an avirulent pathogen and BTH treatment of *B. napus* plants resulted in the rapid accumulation of *PR-1* and *PR-2* transcripts, two genes widely accepted as markers for SAR (28). Comparisons of different treatments indicate that the expression level of these marker genes was associated with the effectiveness of SAR. For example, plants preinoculated with avirulent *P. syringae* pv. *maculicola* 1120B expressed relatively low levels of PR genes in systemic leaves and displayed only moderately higher levels of resistance compared with untreated plants, which did not accumulate detectable levels of PR transcripts prior to secondary infection with virulent pathogens. Plants treated with BTH expressed the highest levels of PR gene mRNA and displayed the most resistance (Figs. 2 to 4). In contrast, *NahG* plants failed to display SAR in response to inoculation with avirulent *P. syringae* pv. *maculicola* 1120B and accumulated substantially less *BnPR-1* gene transcripts in local and systemic leaves (Fig. 5D) and cotyledons (Fig. 6B). The reduction in *BnPR-1* levels compared with untransformed (wild-type) plants was particularly evident in the early stages following preinoculation with the avirulent pathogen. This association of *BnPR* gene transcript accumulation with SAR suggests that the proteins they encode likely contribute to the observed increase in disease resistance.

Hennin et al. (14) reported rapid induction of *PR-1* and *PR-2* transcripts in *B. napus* following treatment with BTH. However, these authors did not correlate PR gene expression with disease resistance. BTH treatment also led to the enhancement of β -1,3-glucanase (*PR-2*) enzymatic activity and protein levels in *B. oleracea* seedlings, before and after infection with *Peronospora parasitica* (38). However, whereas our study and that of Hennin et al. (14) clearly observed rapid and dramatic increases in *BnPR-1* mRNA, Ziadi et al. (38) detected only weak and slow induction of *PR-1*. One possible explanation for this discrepancy is that the *BnPR-1* cDNA used as a probe encodes a protein distinct from the one recognized by the PR-1 antibody, which was raised against a tobacco isoform. Alternatively, the differences may be attributed to posttranscriptional regulation of PR-1 or to the higher levels of BTH used in the present study and in that of Hennin et al. (14). Ziadi et al. (38) also failed to detect substantial changes in the expression of PR-5 protein, which is consistent with our inability to detect *PR-5* mRNA following pathogen challenge or during SAR. Together, these results suggest that PR-5 may not be associated with SAR in *Brassica* species.

Our conclusion that SAR in *B. napus* requires SA is based on two lines of evidence. First, BTH, a structural analog of SA, was highly effective at enhancing resistance to *P. syringae* pv. *maculicola* and *L. maculans*. In other plant species BTH and SA induce the expression of the same set of SAR genes and confer resistance to the same spectrum of pathogens (9,20). Both compounds are thought to activate SAR through the same signaling pathway, possibly by binding to the same receptor(s) (28). Second, blocking the pathogen-induced accumulation of free SA by expression of the *NahG* transgene abolished the effectiveness of the localized preinoculation treatment in conferring resistance against subsequent infections by virulent pathogens. *NahG B. napus* plants continued to mount an effective SAR in response to BTH treatment (Figs. 5C and 6A), indicating that the above effect was largely due to the inability to accumulate SA.

In addition to being unable to mount an effective SAR response, *Arabidopsis* and tobacco *NahG* plants have been reported to be severely compromised in basal resistance (5). In contrast, *NahG B. napus* plants were only marginally more susceptible to virulent *P. syringae* pv. *maculicola* in the absence of SAR-

inducing pretreatments (Fig. 5C), suggesting that loss of SA preferentially compromises SAR, but not basal resistance, against this pathogen in *B. napus*. Also, the reduction of PR gene transcript levels observed in *B. napus NahG* plants and seedlings was much more pronounced in the early stages following infection with the avirulent pathogen (Figs. 5D and 6B). Analysis by GC-MS confirmed the effective block of SA accumulation in *B. napus NahG* plants (Fig. 5B). This suggests an important contribution of one or more SA-independent signaling pathways for the late-onset pathogen-induced accumulation of *PR-1* and *PR-2* transcripts in this species. Further experimentation will be required to test this hypothesis and define the pathways involved. Nevertheless, the observation that delaying the accumulation of PR genes in *NahG B. napus* plants substantially compromised disease resistance is consistent with the notion that the speed at which defense responses are activated is critical in determining the outcome of plant-pathogen interactions (31).

The fact that preinoculation with an avirulent strain of *P. syringae* pv. *maculicola*, and spraying with BTH, induced the expression of *BnPR-1* and *BnPR-2* and conferred resistance to virulent strains of both *P. syringae* pv. *maculicola* and *L. maculans* suggests that both these treatments may activate the same signaling pathways in *B. napus*. This phenomenon was also observed in *Arabidopsis*, tobacco, and other plant species (33,35). However, the same is not true of the chemical MSB, which induced systemic resistance against *L. maculans* without activation of *BnPR-1* (1). Despite the possibility that they may induce the same signaling cascades, our results clearly show that preinoculation with an avirulent pathogen and BTH treatment are not equally effective at conferring SAR. Previous reports suggest that SAR requires an induction time of about 2 days to 2 weeks to be fully established (30). Altering the length of time between the preinoculation and the infection with virulent *P. syringae* pv. *maculicola* did not increase the effectiveness of this pretreatment relative to BTH application (Fig. 2A). Localized infection with a microbe capable of eliciting plant cell necrosis is thought to trigger the production of a systemic SAR signal that is mobilized through the phloem (7,24). The nature of the systemic signal is still unknown, although recent evidence suggests the involvement of a lipid-based molecule (7,19,24). Application of the SA analog INA can rescue SAR in the *Arabidopsis defective in induced resistance1* mutant, which is unable to produce the systemic signal (24), indicating that treatment with INA, and therefore probably BTH as well, likely bypasses the requirement for the systemic signal. Accordingly, the relatively low level of SAR triggered by preinoculation with the avirulent pathogen, compared to BTH treatment, may indicate that the production, mobilization, or perception of the systemic signal is limiting the effectiveness of localized preinoculation with avirulent pathogens. In addition to altering the time between preinoculation and the infection with virulent *P. syringae* pv. *maculicola*, we also tested the effect of altering the strain of avirulent bacterium and the area of tissue preinoculated, but neither substantially increased SAR (data not shown). We do not believe that the superior performance of BTH is due to direct growth inhibition of the virulent pathogens, since neither BTH nor its metabolites appear to have antifungal activities (9). Furthermore, localized infiltration of BTH on uninfected leaves was as effective as spraying the entire plant in eliciting SAR (data not shown).

A previous report had demonstrated that pre- or coinfection with a weakly virulent strain of *L. maculans* was effective at inducing SAR against a highly virulent strain in mature *B. napus* plants (23). Our results confirm that preinfection with an avirulent pathogen is effective at inducing SAR against virulent *L. maculans*. Furthermore, they extend previous findings by demonstrating that the enhanced resistance is associated with PR gene induction and requires SA, and that BTH is highly effective at reducing disease caused by *L. maculans*. Importantly, we show that SAR

against *L. maculans* is effective at the seedling stage, a time at which disease caused by this fungus can be particularly devastating.

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