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A comparison of induced resistance activated by benzothiadiazole, (2R,3R)-butanediol and an isoparaffin mixture against anthracnose of *Nicotiana benthamiana*

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Abstract

Resistance in *Nicotiana benthamiana* was activated by benzothiadiazole (BTH), (2R,3R)-butanediol or PC1, an isoparaffin-based mixture, against anthracnose caused by the hemibiotrophic fungus, *Colletotrichum orbiculare*. BTH, (2R,3R)-butanediol and PC1 reduced the number of lesions per leaf area caused by *C. orbiculare* by 98%, 77% and 81%, respectively. Foliar application of BTH induced expression of genes for the acidic pathogenesis-related (PR) proteins, *NbPR-1a*, *NbPR-3Q* and acidic *NbPR-5*. In contrast, soil application of (2R,3R)-butanediol or PC1 primed expression of genes for the basic PR proteins, *NbPRb-1b*, basic *NbPR-2* and *NbPR-5dB*. These results are consistent with the activation of systemic acquired resistance (SAR) by BTH and induced systemic resistance (ISR) by (2R,3R)-butanediol or PC1, and show that (2R,3R)-butanediol and PC1 can affect gene expression similarly to plant growth-promoting rhizobacteria. However, the effects of (2R,3R)-butanediol and PC1 were not identical. In addition to priming, (2R,3R)-butanediol induced expression of basic *NbPR-2*, whereas PC1 treatment induced expression of both *NbPRb-1b* and basic *NbPR-2*. Although a number of microbial products, such as (2R,3R)-butanediol, have been shown to produce ISR, this is the first demonstration that an isoparaffin-based mixture, which is not derived from a microorganism, can produce ISR.

Keywords: ISR, pathogenesis-related protein, priming, SAR, tobacco, VOC

Introduction

Induced resistance is a physiological state in which environmental, chemical, or biological activators increase a plant's defences against subsequent pathogen challenge. The activator is recognized by the plant, resulting in the induction of defence mechanisms, such as novel gene expression patterns (Edreva, 2004). The two most widely studied types of induced resistance are systemic acquired resistance (SAR) and induced systemic resistance (ISR). For this report, we will use these terms based on the descriptions of Ton et al. (2006), where SAR is an enhancement of salicylic acid (SA)-dependent basal resistance, and ISR is an enhancement of jasmonate/ethylene (JA/ET)-dependent basal resistance.

SAR can be induced in plants by activators produced by bacteria and fungi, such as antibiotics, chitin and β-glucans (Lyon, 2007), or by synthetic activators, such as benzothiadiazole (BTH) (Friedrich et al 1996). ISR can be induced by beneficial soil-borne plant growth-promoting rhizobacteria (PGPR), such as several species of *Pseudomonas* and

Bacillus, that colonize roots but induce resistance systemically (van Loon et al., 1998). PGPR produce certain compounds that can activate ISR, such as volatile organic compounds (VOCs), like acetoin and (2R,3R)-butanediol, that are secreted by *Pseudomonas chlororaphis* O6, *Bacillus amyloliquefaciens* IN937a and *B. subtilis* GB03 (Han et al., 2006; Ryu et al., 2004;). The ability of microbial products to activate ISR is specific as Han et al. (2006) showed that an isomer, (2S,3S)-butanediol, did not affect plant growth or resistance to *Erwinia carotovora* subsp. *carotovora* SCC1 in *Nicotiana tabacum*. In addition to inducing resistance, acetoin and (2R,3R)-butanediol cause plant growth promotion, such as in *N. benthamiana* (Ryu et al., 2005) and *N. tabacum* (Han et al., 2006).

One of the best characterized SAR responses associated with BTH and other SAR activators is the induction of expression of genes for acidic PR proteins that mostly accumulate in the extracellular spaces (van Loon and van Strien, 1999). For example, among the PR protein genes showing induced expression during SAR in *N. tabacum* are those for variants of acidic PR-1 (PR-1a, PR-1b, and PR-1c), acidic β-1,3-glucanases (PR-2a, PR-2b, PR-2c and PR-2d), acidic chitinases (PR-P, PR-Q, PR-4a and PR-4b) and acidic thaumatin-like proteins (PR-R major and PR-R minor (PR-S)) (van Loon and van Strien, 1999).

In contrast, ISR has frequently been associated with accumulation of basic PR proteins, such as PRb-1b, basic PR-2 and PR-5d, which are typically secreted into the vacuole (Alfano et al., 2007; Memelink et al., 1990; Pozo et al., 1999; Spencer et al., 2003). Activation of ISR is associated with priming of gene expression, which is a physiological state in which plants respond more rapidly and strongly upon pathogen attack because their defense genes are expressed more rapidly after pathogen attack (Conrath et al., 2006). Verhagen et al. (2004) found that genes were not induced systemically after treatment with *P. fluorescens* WCS417r but were primed for increased expression when treated *A. thaliana* plants were challenged with *P. syringae* pv. t*omato* DC3000. Some defense genes, however, have been found to be systemically induced rather than primed during ISR. For example, expression of *PRb-1b* and an HMGR gene was induced systemically after root treatment of *N. tabacum* with *P. chlororaphis* O6 (Spencer et al., 2003). Although (2R,3R)-butanediol induces ISR, there have not yet been any studies on whether this PGPR product affects plant gene expression.

Among plants used as models for molecular biology is *N. benthamiana*, which is a close relative of *N. tabacum* (Goodin et al., 2008). A pathogen that infects *N. tabacum* and *N. benthamiana* is the intracellular hemibiotrophic fungus, *Colletotrichum orbiculare*, which causes anthracnose (Shen et al., 2001). The aim of this study was to use the interaction between *C. orbiculare* and *N. benthamiana* to evaluate SAR- and ISR-related gene induction (i.e., increased expression compared to the control after treatment but before pathogen challenge) and priming (i.e., increased expression compared to the control after pathogen challenge) for the well characterized activators, BTH and (2R,3R)-butanediol, along with PC1, which has been developed by Petro-Canada to control plant diseases. PC1 is a clear, colourless liquid at room temperature composed of a mixture of food-grade emulsifiers and food-grade synthetic isoparaffins with a carbon number distribution of approximately 16 to 35 atoms. This active ingredient was registered as the fungicide Civitas™ in early 2009 in the U.S.A.

Materials and Methods

Plant treatments

Nicotiana benthamiana was grown as per Shen et al. (2001), except that plants were grown in Sunshine aggregate plus mix # 4 (SunGro Horticulture Canada Ltd., Seba Beach, AB) for BTH or (2R,3R)-butanediol treatments or a mix with 50% pasteurized sandy loam soil from the Guelph Turfgrass Institute at the University of Guelph for PC1 treatment. Pasteurization was done at 60ºC for 30 min.

Nicotiana benthamiana plants at the 2nd true-leaf stage were treated by spraying with 1.2 mM BTH (Actigard 50WG, Syngenta, Guelph, ON) until run-off on both the abaxial and adaxial sides of the leaves or by pipetting onto the soil either 10 ml 100 μM (2R,3R)-butanediol (Sigma-Aldrich, Oakville, ON) per pot or 15ml 10% or 20% PC1 (Petro-Canada, Mississauga, ON) per pot. Control plants were likewise treated with distilled water. The plants were then used for disease assessment and analysis of gene expression.

Fungal inoculation and disease assessment

Colletotrichum orbiculare ATCC20767P1 (Chen et al., 2003) was grown on sodium chloride–yeast extract–sucrose agar medium (SYAS) (Shen et al. 2001) at 22ºC under continuous fluorescent light. After 10 d, conidia of *C. orbiculare* were washed with sterile deionized water.

Nicotiana benthamiana was inoculated at 168 h after BTH, (2R,3R)-butanediol or PC1 treatment with *C. orbiculare* at $2x10^6$ conidia/ml using an art spray brush connected to an air compressor (Model FP200300AV, Campbell Hausfeld, Harrison, OH). Inoculated plants were immediately enclosed in 17 L black plastic–lined containers under high humidity and incubated at room temperature.

The level of disease was determined by counting the number of lesions per leaf at 96 hours post inoculation (HPI), and measuring the area of each leaf using a leaf area meter (Model 3100, LI-COR, Lincoln, NB). The lesions per cm^2 were then calculated. For each treatment (BTH, butanediol or PC1) and its corresponding water control, means were pooled from three separate experiments with a total of nine replications. An Analysis of Variance using Dunnett's method was used to separate means.

Antimicrobial activity test

Plugs, 5 mm in diameter, of *C. orbiculare*, were grown on potato dextrose agar (PDA, Fisher Scientific, Mississauga, ON), and transferred to PDA amended with 0, 0.1, 1.0 or 10 mM BTH, or PDA amended with 0, 1, 5, 10, or 20% (v/v) PC1. PDA plugs were also placed on PDA plates, which were exposed to 1 mL solutions of 0, 10, 100 or 1000 μM (2R,3R)-butanediol placed on upturned Petri plate lids, and sealed with parafilm. There were four replicate plates per treatment. Plates were incubated at room temperature up to 9 d, and radial growth was measured daily. Growth inhibition was calculated as (growth on control plate - growth on amended plate) / (growth on control plate).

Collection of samples and RNA extraction

The two youngest fully-developed leaves of *N. benthamiana*-treated with BTH were harvested at 0, 12. 24, 48, 72 and 168 h post treatment (HPT). Similarly, the two youngest fully-developed leaves of *N. benthamiana* treated with (2R,3R)-butanediol or PC1 and then inoculated with *C. orbiculare* were harvested at 0, 72 and 168, 192, 216, 240 and 264 HPT. Fungal inoculation occurred at 168 HPT, and 192, 216, 240 and 264 HPT, which corresponded to 24, 48, 72, and 96 h post inoculation (HPI), respectively. All samples were immediately frozen at -80°C for later RNA extraction following Xie et al (2009).

Primer design

For SAR and ISR assessment in *N. benthamiana*, previously published SAR-induced or ISR-primed gene sequences (Table 1) from *N. benthamiana*, *N. tabacum* or *Solanum lycopersicum* obtained from the NCBI GenBank NR database (http://www.ncbi.nlm.nih.gov/) were used as query sequences in a BLASTN search using The Computational Biology and Functional Genomics Laboratory Gene Indices for *N. benthamiana* and *N. tabacum* (http://compbio.dfci.harvard.edu/index.html) and the GenBank NR databases. Sequences with an e-value lower than 10^{-10} were collected from *N. benthamiana* and other *Nicotiana* species and aligned with MUSCLE (Edgar, 2004), and primers were designed in unique regions using GeneRunner (Hastings Software, Hastings, NY) (Table 2).

Relative RT-PCR

Single-stranded cDNA was synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON) and oligo (dT) primer with total RNA following the manufacturer's instructions. Transcript levels were determined by relative RT-PCR, which involves co-amplification of the gene of interest with a constitutively expressed control gene, the translation elongation factor–1α (*NbEF-1α*), this is used as a standard for comparison of band intensity (Dean et al., 2002).

Primer sequences for *NbPR-1a* and basic *NbPR-2* described by Dean et al. (2002) and primer sequences for *NbPR-4* (that actually amplified *NbPR-3Q*) described by Seo et al. (2006) were used. Primers were designed for a homolog of the acidic PR-5 gene (X12739) of *N. tabacum* that was up-regulated in leaves after TMV was used as activator of SAR (Payne et al., 1988). To design primers for acidic *NbPR-5*, an alignment was made of X12739 with genes for an acidic PR-5 of *N. benthamiana* (TC8513) and acidic PR-5s (X03913, X15223 and TC6745) of *N. tabacum*. Also included was a methionine synthase sequence from *N. benthamiana* (TC14297), which had some but much less amino acid sequence homology to X12739 than the acidic PR-5s. The forward primer was designed in a conserved region between all these PR-5 sequences but different from the methionine synthase gene, while the reverse primer was designed in conserved region between all the acidic PR-5 sequences but divergent from basic PR5 sequences of *N. benthamiana* (TC8181 and TC7790) and the methionine synthase gene. Primers were also designed for a homolog of *PRb-1b* of *N. tabacum* (X14065), which is induced in ethephon-treated leaves (Memelink et al. 1990). The forward and reverse primers for *NbPRb-1b* were designed in a conserved region between X14065 and its homolog in *N. benthamiana* (TC7078), but divergent from several *PRb-1b*–like genes from *N. benthamiana* (TC9981, TC7080 and CK298472). These primers were in divergent regions compared to *NbPR-1a* (Dean et al., 2002). Lastly, primers were designed for a homolog of the neutral osmotin gene, *PR-5dB* (TC116429) of *S. lycopersicum* that is upregulated in leaves after root treatment with the ISR-inducing fungus, *Trichoderma hamatum* 382 (Alfano et al., 2007). Forward and reverse primers for *NbPR-5db* were designed in conserved regions between TC116429 and the matching *N. benthamiana* (TC8181) and *N. tabacum* (TC4103 and TC4068) sequences, but divergent from other *N. benthamiana* (TC7790) and *N. tabacum* (TC12961) sequences.

RT-PCRs were done in 15 µl reaction volumes with 2 µl cDNA, 0.75 units *Tsg* polymerase (Biobasic, Toronto, ON), 10x *Tsg* polymerase buffer, 2 mM dNTPs, and 2.5 mM Mg^{2+}, with 1.0 mM of each primer (Dean et al., 2002). Amplification conditions consisted of one cycle at 94ºC for 3 min followed by 30 cycles of 94ºC for 30 s, 55ºC, 60ºC or 65ºC for 1 min, and 72ºC for 1 min, with a final extension period of 10 min at 72ºC. The annealing temperature was 55ºC for *NbPR-1a*, 60ºC for basic *NbPR-2*, acidic *NbPR-5* and *NbPRb-1b*, and 65ºC for *NbPR-3Q* and *NbPR5-dB*, To confirm that the relative expression values were within the number of cycles that gives accurate quantification, relative RT-PCR was done with 25 cycles, which is 5 cycles fewer than the number used for quantification. The expression pattern was compared to that obtained with 30 cycles to show that the pattern of expression was not significantly different for each gene. All reactions were carried out in an Eppendorf AG 22331 Master Cycler (Eppendorf, Hamburg, DE).

The RT-PCR products were separated in 1% TAE agarose gels and stained with ethidium bromide. Bands were visualized with an UV transilluminator, and gel pictures were saved as TIFF electronic image files, and imported into NIH Image (Scion Corporation, Frederick, MD) for quantification. In each lane, the band intensities were determined for both the gene of interest and the relative control, *NbEF-1*α. The relative expression for the gene of interest was calculated by taking a ratio of its band intensity over the band intensity of *NbEF-1*α (Dean et al., 2002). Identity of RT-PCR products was confirmed by direct sequencing of the respective RT-PCR product at the Laboratory Services Division, (University of Guelph,

Guelph, ON). To confirm the identity of each sequenced RT-PCR product, a BLASTN search on The Computational Biology and Functional Genomics Laboratory Gene Index databases for *N. tabacum* and *N. benthamiana*, and the NCBI GenBank NR database was done using the sequenced RT-PCR products as the query sequence.

Results

Disease assessment of *N. benthamiana* treated with BTH, butanediol or PC1.

At 96 HPI with *C. orbiculare* in BTH treated plants, the number of lesions/cm^2 was 50 times lower than that for water-treated plants (Table 3). For (2R,3R)-butanediol treated plants, the number of lesions/cm^2 was 4.5 and 4.25 times lower, respectively, than plants treated with water or (2S,3S)-butanediol, and the number of lesions/cm^2 in 10% or 20% PC1-treated plants was 3.5 to 5.25 times lower than for water-treated plants (Table 3). The timing of the appearance of symptoms and the appearance of individual lesions did not differ between the plants treated with water, BTH, (2R,3R)-butanediol, or PC1. As there was no significant difference between the number of lesions/cm^2 in the plants treated with 10% PC1 or 20% PC1, subsequent analysis of gene expression after PC1 treatment was done using 10% PC1.

BTH, (2R,3R)-butanediol and PC1 antimicrobial activity

An examination of the direct antifungal activity of 0, 0.1, 1.0 and 10 mM BTH showed that growth of *C. orbiculare* was affected by higher levels of BTH. Compared to the control, the concentration of BTH that inhibited growth by 50% (EC_{50}) was 15.1 mM measured from day 3 to 9 on agar medium. On PDA alone and 0.1 mM BTH, the mycelium was dark with masses of orange conidia near the edges of the colony, but at 1.0 mM BTH, the hyphae appeared to be sparser in the center with more conidia. This was not observed at 10 mM, where the only visible effect of BTH was a reduction in fungal colony size. In contrast, there was no visible effect on fungal growth or morphlogy when the fungus was grown on 10, 100 or 1000 μM (2R,3R)-butanediol.

Some antifungal activity of 1, 5, 10, and 20% PC1 was observed against *C. orbiculare*. Compared to the control, the percentages (vol/vol) at which PC1 inhibited fungal growth by 50% (EC_{50}) were 15.8% from 1-4 days, 21.2% from 4-7 days and >100% from 7-10 days. The major change in EC_{50} values after one week occurred because PC1 was no longer affecting the growth rate by that time, and radial growth on 20% PC1 was as fast as the unamended control. On PDA alone and 5% PC1, the mycelium was dark with masses of orange conidia, but at 10% or 20% PC1 in PDA, the hyphae was dark but appeared water soaked (not fluffy nor aerial), and there was increased production of orange conidia

Gene expression after BTH treatment

After a 12 h period of no change in expression after BTH treatment, expression of *NbPR-1a* was rapidly induced, peaking at 120 HPT (Fig 1). After that, it remained relatively unchanged. No expression was detected with the water control.

While a RT-PCR product of the predicted size was obtained for *NbPR-4*, sequence analysis showed that the band for *NbPR-4* actually matched *NbPR-3Q* (TC7119) (100% nt identity compared to less than 50% nt identity with *NbPR4*). Therefore, the primers were amplifying *NbPR-3Q*, and thus, this PCR product is described as *NbPR-3Q* rather than *NbPR-4*. Expression of *NbPR-3Q* was induced significantly by 12 HPT without the lag period observed with *NbPR-1a* (Fig 1). Expression then continued to rapidly increase, peaking at 72 HPT. After that, expression fluctuated but remained relatively unchanged. Expression levels of *NbPR-3Q* in the water control did not change over pre-treatment levels.

Expression of acidic *NbPR-5* was similar to *NbPR-1a* in that it showed a lag period of 12 h after treatment before being induced by the BTH treatment (Fig 1). However, unlike *NbPR-1a*, the rapid increase of expression of acidic *NbPR-5* peaked at 72 HPT and was

followed by a rapid decline by 120 HPT, after which the expression level remained stable and higher than the pre-treatment level. No expression was detected with the water control.

Expression of three genes believed to be involved in ISR, *NbPRb-1b*, basic *NbPR-2* and *NbPR5-dB*, that are described below were also tested after BTH treatment (Fig. 1). Expression of these genes in both the BTH-treated and water-treated plants slightly increased, but there was no significant induction over the controls. The expression of these three genes also increased to a similar extent after inoculation for both the BTH-treated and water control plants.

Gene expression after (2R,3R)-butanediol treatment

There was a slight induction of *NbPRb-1b* expression at 72 HPT for the control and all treatments (Fig 2). After *C. orbiculare* inoculation, increased expression of *NbPRb-1b* was observed in plants treated with water and (2S,3S)-butanediol, but this was much less than in plants treated with (2R,3R)-butanediol beginning at 192 HPT with a peak expression at 240 HPT, which was the same time that expression peaked in the controls. This indicates that *NbPRb-1b* expression was being primed by (2R,3R)-butanediol after inoculation.

A gradual induction of basic *NbPR-2* expression for the (2R,3R)-butanediol treatment resulted in expression levels significantly higher than the water and (2S,3S)-butanediol controls at 72 and 168 HPT (Fig 2). After inoculation with *C. orbiculare*, basic *NbPR-2* transcript levels continuously increased in (2R,3R)-butanediol-treated plants until 264 HPT, which was significantly higher than that of the controls, except at 192 HPT, indicating priming. In addition to a smaller increase in expression, expression in the control treatments peaked at 240 HPT, whereas expression continued to increase for (2R,3R)-butanediol.

Expression of *NbPR5-dB* gradually increased similarly after treatment with (2R,3R)-butanediol, (2S,3S)-butanediol or water (Fig 2). After inoculation with *C. orbiculare*, transcript levels were significantly more up-regulated by 240 HPT in plants treated with (2R,3R)-butanediol than either water or (2S,3S)-butanediol, indicating priming. Expression in plants treated with (2R,3R)-butanediol became much greater than that of the controls by 264 HPT.

For the three SAR inducible genes described above, no detection of *NbPR-1a* and acidic *NbPR-5*, and no increase in low levels of *NbPR-3Q* expression were found after (2R,3R)-butanediol, (2S,3S)-butanediol or water control treatments prior to fungal inoculation (Fig. 2). Expression of these genes increased after *C. orbiculare* inoculation in the three treatments, but there were no significant differences between each treatment. This indicates that none of the SAR induced genes responded to (2R,3R)-butanediol or (2S,3S)-butanediol, but they all respond to pathogen attack.

Gene expression after PC1 treatment

Transcript levels of *NbPR-1a*, *NbPR-3Q* and acidic *NbPR-5* did not increase after PC1 treatment (Fig 3). This indicates that none of the SAR-induced genes responded to PC1 treatment, which is similar to the results observed in plants treated with (2R,3R)-butanediol.

Application of PC1 resulted in a significant induction of *NbPRb-1b* expression over water-treated plants by 168 HPT (Fig 3). After inoculation with *C. orbiculare*, *NbPRb-1b* expression increased significantly more in PC1-treated plants than in water-treated plants, peaking at 240 HPT, indicating that priming also occurred.

There was a significant induction of basic *NbPR-2* expression in PC1-treated plants compared to water-treated plants by 72 HPT (Fig 3). After inoculation with *C. orbiculare*, basic *NbPR-2* expression increased significantly more in PC1-treated plants than in water-treated plants peaking at 240 HPT, like *NbPRb-1b*. Thus, both PC1 and (2R,3R)-butanediol induced and primed expression of basic *NbPR-2* after inoculation.

Expression of *NbPR5-dB* after PC1 treatment was not greater than the water control prior to inoculation, indicating that the gene was not induced. After inoculation, expression of

NbPR5-dB was primed, with a significantly higher expression than water-treated plants (Fig 3). Unlike *NbPRb-1b* and basic *NbPR-2*, expression of *NbPR5-dB* did not peak prior to the end of the experiment.

Discussion

Anthracnose disease severity caused by *C. orbiculare* in *N. benthamiana* was reduced by both BTH and (2R,3R)-butanediol. This shows that both SAR and ISR activators are effective against this hemibiotrophic pathogen. In addition, a mixture of food-grade isoparaffin compounds, PC1, also reduced lesion numbers. Although PC1 caused a reduction in growth rate of *C. orbiculare*, this effect was only temporary (within the first week). By comparison, the known SAR activator, BTH, also inhibited the growth rate of *C. orbiculare*, but the effect was not temporary like that of PC1. It thus appeared that the effect of PC1 on disease reduction could be due to inducing resistance in the plants.

One of the most widely studied mechanisms of induced resistance is SAR. In SAR, the expression of genes linked to the SA pathway, such as acidic members of the pathogenesis related (PR) proteins, have been shown to increase after treatment with SA, BTH or biological activators, like TMV (Friedrich et al., 1996; van Loon and van Strien, 1999). For example, BTH highly induced expression of *PR-1a*, *PR-3Q*, and acidic *PR-5* in *N. tabacum* (Friedrich et al., 1996).

Acidic PR-1a is the most abundantly expressed gene after biological or chemical induction of SAR, and therefore is one of the most commonly used markers for SAR (van Loon and van Strien, 1999). Although a clear function of PR-1a has not been yet detected, PR-1 proteins were associated with host cell wall outgrowths and papillae produced by tobacco roots in response to *Chalara elegans*, indicating a role in increasing mechanical strength of these defense-related structures (Tahiri-Alaoui et al., 1993). Our results show that *NbPR-1a* expression was significantly induced after BTH treatment following a lag period of 12 hours. This is similar to *NtPR-1a* expression in *N. tabacum* after BTH treatment (Friedrich et al., 1996), where expression increased after a lag of 12 hours. *NbPR-1a* expression was not detected in healthy leaves prior to treatment, just as in healthy *N. tabacum*, where *NtPR-1a* expression was undetectable (Friedrich et al., 1996). These similarities are not surprising since *NbPR-1a* has 96% nt identity to *NtPR-1a*.

The PR-3 protein family is one of four different families of PR proteins classified as chitinases (Datta and Muthukrishnan, 1999). Chitinases are endo β-1,4-glucosaminidases, which hydrolyze the β-glycosidic bond of chitin in the fungal cell wall (Schlumbaum et al., 1986). Based on sequence similarity and presence of a chitin binding domain (CBD), five subclasses of the PR-3 proteins have been identified (Melchers et al., 1994). NbPR-3Q belongs to PR-3 subclass II enzymes that lack the CBD, which is also found with the acidic PR-3 proteins in *N. tabacum* (Neuhaus, 1999). Acidic PR-3 proteins in *N. tabacum* are further divided into acidic *PR-Q* and acidic *PR-P* (Datta and Muthukrishnan, 1999). *NtPR-Q* was induced after treatment with SA, BTH or TMV to induce SAR (Friedrich et al, 1996). In this study, *NbPR-3Q* expression was observed in healthy plants and induced by 12 HPT with BTH remaining induced during the remainder of the experiment. Similarly, Friedrich et al. (1996) reported increased levels of *NtPR-Q* mRNA at 12 HPT with BTH continuing until 20 days post treatment. The SA- and BTH-inducible *NtPR-Q* has 96% nt identity to the BTH-inducible *NbPR-3Q*.

PR-5 proteins with an acidic pI are called thaumatin-like proteins (Datta and Muthukrishnan, 1999). The function of PR-5 proteins is not yet known, but they can alter the permeability of fungal membranes, causing lysis (Vigers et al., 1992). In this study, acidic *NbPR-5* expression was not observed prior to treatment but increased after a lag period of 12 h and then declined after 72 HPT to low levels. Friedrich et al. (1996) also showed no acidic

NtPR-5 (PR-R-major) expression in *N. tabacum* cv. Xanthi-nc prior to BTH treatment, but the increase in *NtPR-5* transcripts that peaked at 72 HPT with BTH continued for up to 20 d post treatment. These results suggest that expression of acidic PR-5 after BTH application persisted for a much shorter period in *N. benthamiana* than in *N. tabacum*. Acidic *NtPR-5* has 90% nt identity to acidic *NbPR-5*.

In this study, no induction of three ISR-related genes, *NbPRb-1b*, basic *NbPR-2* and *NbPR-5dB*, was observed after BTH treatment. Molina et al. (1999) also showed that *NtPRb-1b* was not induced after BTH treatment. Expression of basic *NtPR-2* was only slightly induced by BTH treatment over a high background level (Friedrich et al., 1996).

Although it is known that the rhizobacterial VOC, (2R,3R)-butanediol, contributes to resistance related to PGPRs that induce ISR (Ryu et al., 2004), it is not known if it affects plant gene expression like that caused by PGPRs. An examination of known signalling pathways in *A. thaliana* showed that ISR triggered by (2R,3R)-butanediol was independent of SA and JA signalling pathways but was mediated by the ET pathway (Ryu et al., 2004). Increased expression or priming of genes linked with the ET and/or JA signalling pathway have been observed after root colonization by ISR inducing PGPRs and mycorrhizae (van Loon and Bakker, 2006). For example, root treatment with *P. chlororaphis* O6 induced basic *PRb-1b* in *N. tabacum* leaves (Spencer et al., 2003), root treatment with *Glomus mosseae* primed expression of two basic PR-2 genes in leaves of *S. lycopersicum* (Pozo et al., 1999), and root treatment with *T. hamatum* 382 induced expression of basic PR-5 in leaves of *S. lycopersicum* (Alfano et al., 2007).

No function has been yet detected for basic PR-1 proteins, which are regulated by the ET pathway (Memelink et al., 1990; van Loon and van Strien, 1999). However, constitutive expression of a pepper basic *PR-1* gene, *CABPR1*, in *N. tabacum* cv. Xanthi resulted in increased disease resistance (Sarowar et al., 2005). In this study, *NbPRb-1b* expression was not induced with (2R,3R)-butanediol but was primed as it became much higher after *C. orbiculare* inoculation in treated plants than in the controls. However, expression of *NtPRb-1b* was induced in *N. tabacum* leaves by 7 to 10 days after exposure of roots to the ISR-inducing PGPR, *P. chlororaphis* O6 (Spencer et al., 2003). Priming was not tested in their experiments. *NtPRb-1b* is very similar to *NbPRb-1b* having 94% nt identity.

Basic β-1, 3-glucanases belong to the class I PR-2 family and catalyze endo-type hydrolytic cleavage of β-1, 3-glucans, which are components of fungal cell walls (Datta and Muthukrishnan, 1999). Basic *NbPR-2* expression was significantly induced by 72 HPT with (2R,3R)-butanediol similar to basic *NtPR-2* which was strongly induced within 2 days after ET treatment (Brederode et al., 1991). Basic *NtPR-2* and *NbPR2* have high (97%) nt identity. Expression of basic *NbPR-2* was also primed in (2R,3R)-butanediol-treated plants increasing significantly more than in the controls after *C. orbiculare* inoculation. Pozo et al. (1999) showed that there was no induction but there was priming of two basic PR-2 genes after treatment of *S. lycopersicum* roots with the mycorrhizal fungus, *G. mosseae*.

The neutral forms of the PR-5 family, like *NtPR-5d* in *N. tabacum*, are classified as osmotin-like proteins (Datta and Muthukrishnan, 1999). *NtPR-5d* showed inhibitory activity against several phytopathogenic and non-phytopathogenic fungi in culture, probably by altering the permeability of fungal membranes, like its acidic counterpart (Vigers et al., 1992). *NbPR-5dB* expression was not induced by (2R,3R)-butanediol but was primed by (2R,3R)-butanediol after *C. orbiculare* inoculation. The priming of this gene is not surprising as the ET pathway regulates ISR (Okubara and Paulitz, 2005), and Xu et al. (1994) showed ET and JA could induce *NtPR-5d* expression by 24 HPT. *NtPR-5d* and *NbPR-5dB* have 94% nt identity.

No induction of any of the SAR-related genes, *NbPR1-a*, *NbPR-3Q* and acidic *NbPR-5*, was observed after (2R,3R)-butanediol treatment. Spencer et al. (2003) did not find

activation of *NtPR-1a in N. tabacum* plants treated with the ISR inducing PGPR, *P. chlororaphis* O6, and León-Kloosterziel et al. (2005) showed that an acidic *PR-5* from *A. thaliana* was not systemically induced after colonization of roots by a non-pathogenic *Pseudomonas* species. As *NbPR1-a*, *NbPR-3Q* and acidic *NbPR-5* can be induced by BTH, which acts through the SA pathway, while the activity of (2R,3R)-butanediol is linked to ET pathway (Han et al., 2006), it is not surprising that they would not be affected by (2R,3R)-butanediol in *N. benthamiana*.

PC1 does not appear to be inducing SAR as there was no up-regulation of the three SAR responsive genes after PC1 treatment matching the results with the (2R,3R)-butanediol treatment. In contrast, *NbPRb-1b* and basic *NbPR-2* were significantly induced prior to inoculation, and the expression of *NbPRb-1b*, basic *NbPR-2* and *NbPR-5dB* was primed after *C. orbiculare* inoculation. Thus, PC1 appears to be associated with ISR since it is inducing and priming genes in a similar way as the (2R,3R)-butanediol.

While both (2R,3R)-butanediol and PC1 appeared to induce ISR, there were some differences between them. (2R,3R)-butanediol treatment only primed *NbPRb-1b* and *NbPR-5dB* expression but both induced and primed expression of basic *NbPR-2*. However, both *NbPRb-1b* and basic *NbPR-2* were induced and primed after PC1 treatment, while *NbPR-5dB* expression was only primed. Thus, PC1, but not (2R,3R)-butanediol, can induce *NbPRb-1b* expression, which may reflect differences in how these chemicals produce ISR in the plant. The mechanism by which PC1 is causing ISR remains to be determined. However, it is possible that PC1 is affecting some aspect of the soil and/or rhizosphere microorganisms related to ISR as foliar PC1 application resulted in damage to the leaves and greater susceptibility to *C. orbiculare* infection, whereas soil application resulted in enhanced resistance to *C. orbiculare* and priming of gene expression in the leaves.

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Table 1 Sequences used in multiple alignments to design primers for assessing expression of genes related to SAR or ISR in *Nicotiana benthamiana*.

Target gene	Gene name	Sequence ID[a]	Species
NbPR-1a	*NbPR-1a*	NA	*N. benthamiana*
NbPR-3Q[b]	*NbPR-4*	NA	*N. benthamiana*
Acidic *NbPR-5*	*NtPR-5R*	X12739[c]	*N. tabacum*
Acidic *NbPR-5*	*NbPR-5*	TC8513[d]	*N. benthamiana*
Acidic *NbPR-5*	*NbMS*	*TC14297*	*N. benthamiana*
Acidic *NbPR-5*	*NtPR-5*	*X03913*	*N. tabacum*
Acidic *NbPR-5*	*NtPR-5S*	*X15223*	*N. tabacum*
Acidic *NbPR-5*	*NtPR-5S*	*TC6745*	*N. tabacum*
NbPRb-1b	*NtPRb-1b*	X14065[e]	*N. tabacum*
NbPRb-1b	*NbPRb-1b*-like	CK298472	*N. benthamiana*
NbPRb-1b	*NbNB*-domain	TC9981	*N. benthamiana*
NbPRb-1b	*NbPRb-1b*-like	TC7080	*N. benthamiana*
NbPRb-1b	*NbPRb-1b*	TC7078[f]	*N. benthamiana*
Basic *NbPR-2*	Basic *NbPR-2*	NA	*N. benthamiana*
NbPR-5dB	*LePR-5*	TC116429[e]	*S. lycopersicum*
NbPR-5dB	*NbPR-5*- like	TC8181[f]	*N. benthamiana*
NbPR-5dB	*NtPR-5*	TC4103	*N. tabacum*
NbPR-5dB	*NtOsm*-like	TC4068	*N. tabacum*
NbPR-5dB	*NtOsm*- like	TC12961	*N. tabacum*
NbPR-5dB	*LePR-5*-like	TC170583	*S. lycopersicum*
NbPR-5dB	*NbOsm*	TC7790	*N. benthamiana*

[a] Sequences obtained from The Computational Biology and Functional Genomics Laboratory Gene Index (http://compbio. dfci. harvard. edu/index. html) are identified as TC sequences. Other sequences were obtained from the NCBI GenBank NR database (http://www. ncbi. nlm. nih. gov/). NA indicates that no sequence was available, but the primer sequences for the target gene was available.

[b] Primer sequences obtained from Seo et al. (2006) that had 100% identity with genes from *N. tabacum PR-Q* (X51425) and *PR-2* (AF141653), and the target gene was renamed *NbPR-3Q*.

[c] Previously published SAR-induced gene sequences used as query sequences in a BLASTN search in the different databases to obtain the other sequences for primer design.

[d] *N. benthamiana* sequences used to design the SAR primers.

[e] Previously published ISR-induced gene sequences used as query sequences in a BLASTN search in the different databases to obtain the other sequences for primer design.

[f] *N. benthamiana* sequences used to design the ISR primers.

Table 2 Primers used for the amplification of genes from *N. benthamiana* in this study.

Gene name	Primers name	Primers	Product size
NbEF-1α	NbEF1α_F630 NbEF1α_R925	F: 5'-TACAACCCTGACAAGATTCC-3' R: 5'-GAGCTTCGTGGTGCATCT-3'	295 bp
NbPR-1a	NbPR1aF NbPR1aR	F: 5'-TGGSATTTRTTCTCTTTTCAC-3'[1] R: 5'-CCTGGAGGATCATAGTTGC-3'	478 bp
NbPR-4	NbPR4F NbPR4R	F: 5'-ATGGAGTTTTCTGGATCACC-3' R: 5'-CTAGCCCTGGCCGAAGTT-3'	745 bp
Acidic *NbPR-5*	NbPR5_F164 NbPR5_R817	F: 5'-GGCAGGCAGCTCAACTCG-3' R: 5'-CGAACAAGAGAATCTGACCCAC-3'	653 bp
NbPRb-1b	NbPR1b1b_F20 NbPR1b-1b_R431	F: 5'-GTTGCTTGTTTCATTACCTTTGC-3' R: 5'-GGTGGATCATAATTGCATGTT-3'	411 bp
Basic *NbPR-2*	NbPR2F NbPR2R	F: 5'-CATCACAGGGTTCGTTTAGGA-3' R: 5'-GGGTTCTTGTTGTTCTCATCA-3'	442 bp
NbPR5-dB	NbPR5dB_F79 NbPR5dB_R481	F: 5'-ACTTATGCTTCCGGCGTA-3' R: 5'-GCACCAGGGCATTCACCA-3'	402 bp

S=G and C; R=A and G.

Table 3 Effect of the SAR-inducing agent BTH or the ISR-inducing agents. (2R,3R)-butanediol or PC1, on disease severity caused by *Colletotrichum orbiculare* on *Nicotiana benthamiana*.

Treatment	Disease severity[a] (Lesions/cm^2)
Control for BTH[b]	2. 50 a
1.2 mM BTH[b]	0. 05 b
Control for butanediol[c]	1. 8 a
100 μM (2S,3S)-butanediol[c]	1. 7 a
100 μM (2R,3R)-butanediol[c]	0. 4 b
Control for PC1[d]	2. 1 a
10% PC1[d]	0. 6 b
20% PC1[d]	0. 4 b

[a]At 7 d post treatment, treated plants were inoculated with 2x10^6 conidia/ml of *C. orbiculare* until run-off, and disease severity was assessed 96 HPI. Means for each treatment and its corresponding water control followed by a letter in common are not significantly different.
[b] Leaves of plants at the two-true-leaf stage were treated with 2 ml on the abaxial and 2 ml on the adaxial surfaces with water (control for BTH) or 1. 2 mM BTH.
[c] Soil of plants at the two true leaf stage were treated with 10 ml water (control for butanediol), 100 μM (2R,3R)-butanediol or (2S,3S)-butanediol (inactive isomer).
[d] Leaves of plants at the two-true-leaf stage were treated with 10 ml of water (control for PC1) or PC1 at 10% or 20%.

Figure Legends

Fig. 1: Relative RT-PCR of the SAR-related genes (*NbPR-1a*, *NbPR-3Q* and acidic *NbPR-5*) and ISR-related genes (*NbPRb-1b*, basic *NbPR-2* and *NbPR-5dB*) in *N. benthamiana* after treatment with 1. 2 mM BTH or water. The quantity of the RT-PCR product was determined relative to that of *NbEF-1α*. Means are shown with standard error bars that were calculated based on four replications. To determine if ISR-related genes were primed after inoculation, plants were inoculated with $2x10^6$ conidia/ml of *C. orbiculare* at 168 HPT.

Fig. 2: Relative RT-PCR of the SAR-related genes (*NbPR-1a*, *NbPR-3Q* and acidic *NbPR-5*) and ISR-related genes (*NbPRb-1b*, basic *NbPR-2* and *NbPR-5dB*) in *N. benthamiana* after treatment with with 100 µM (2R,3R)-butanediol, 100 µM (2S,3S)-butanediol or water. The quantity of the RT-PCR product was determined relative to that of *NbEF-1α*. Means are shown with standard error bars that were calculated based on four replications. To determine if genes were primed after inoculation, plants were inoculated with $2x10^6$ conidia/ml of *C. orbiculare* at 168 HPT.

Fig. 3 Relative RT-PCR of the SAR-related genes (*NbPR-1a*, *NbPR-3Q* and acidic *NbPR-5*) and ISR-related genes (*NbPRb-1b*, basic *NbPR-2* and *NbPR-5dB*) in *N. benthamiana* after treatment with 10% PC1 or water. The quantity of the RT-PCR product was determined relative to that of *NbEF-1α*. Means are shown with standard error bars that were calculated based on four replications. To determine if ISR-related genes were primed after inoculation, plants were inoculated with $2x10^6$ conidia/ml of *C. orbiculare* at 168 HPT.

Fig 1

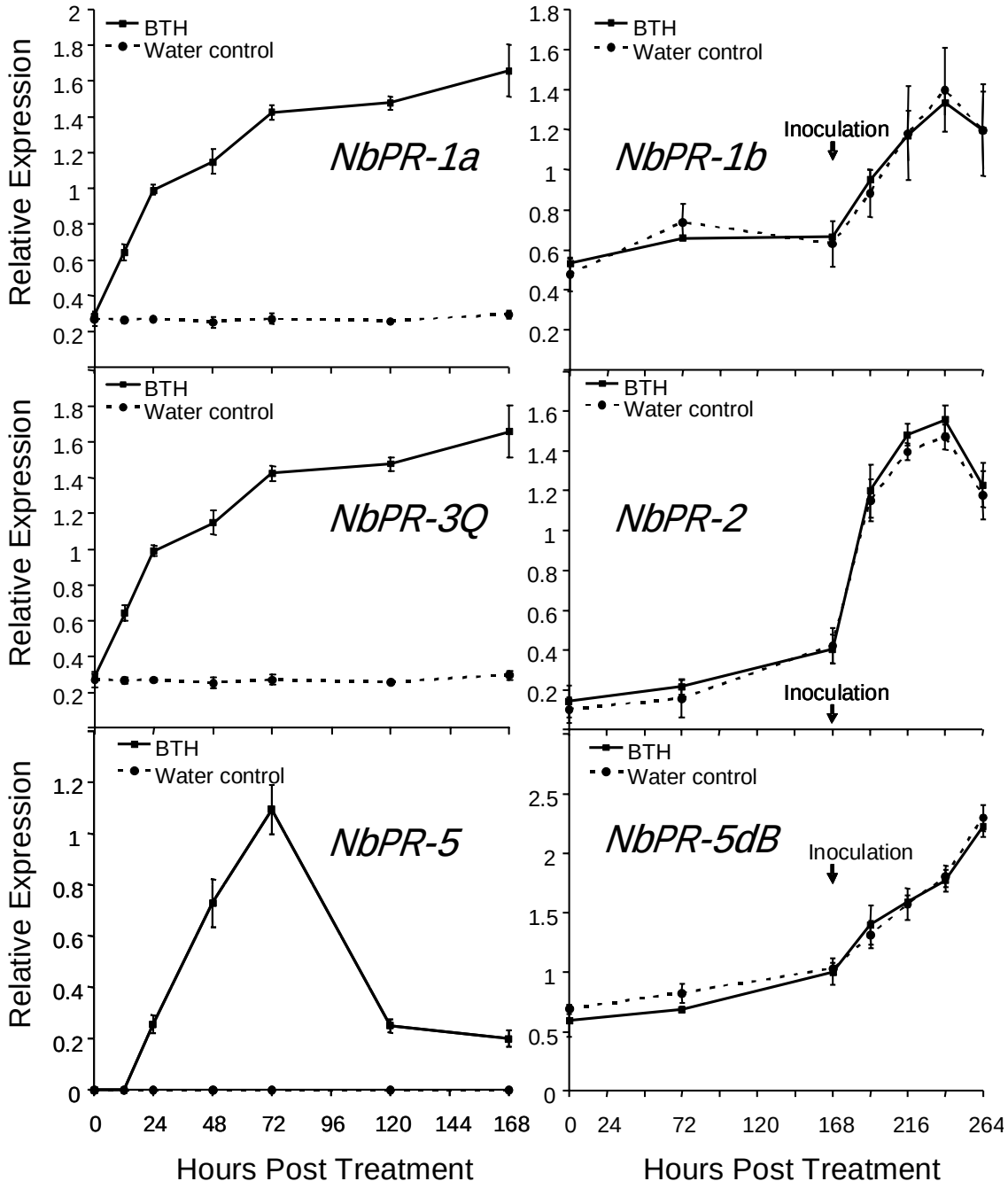

BTH
Water control
Relative Expression
NbPR-1a
NbPR-1b
Inoculation
NbPR-3Q
NbPR-2
NbPR-5
NbPR-5dB
Hours Post Treatment
Hours Post Treatment

Fig. 2

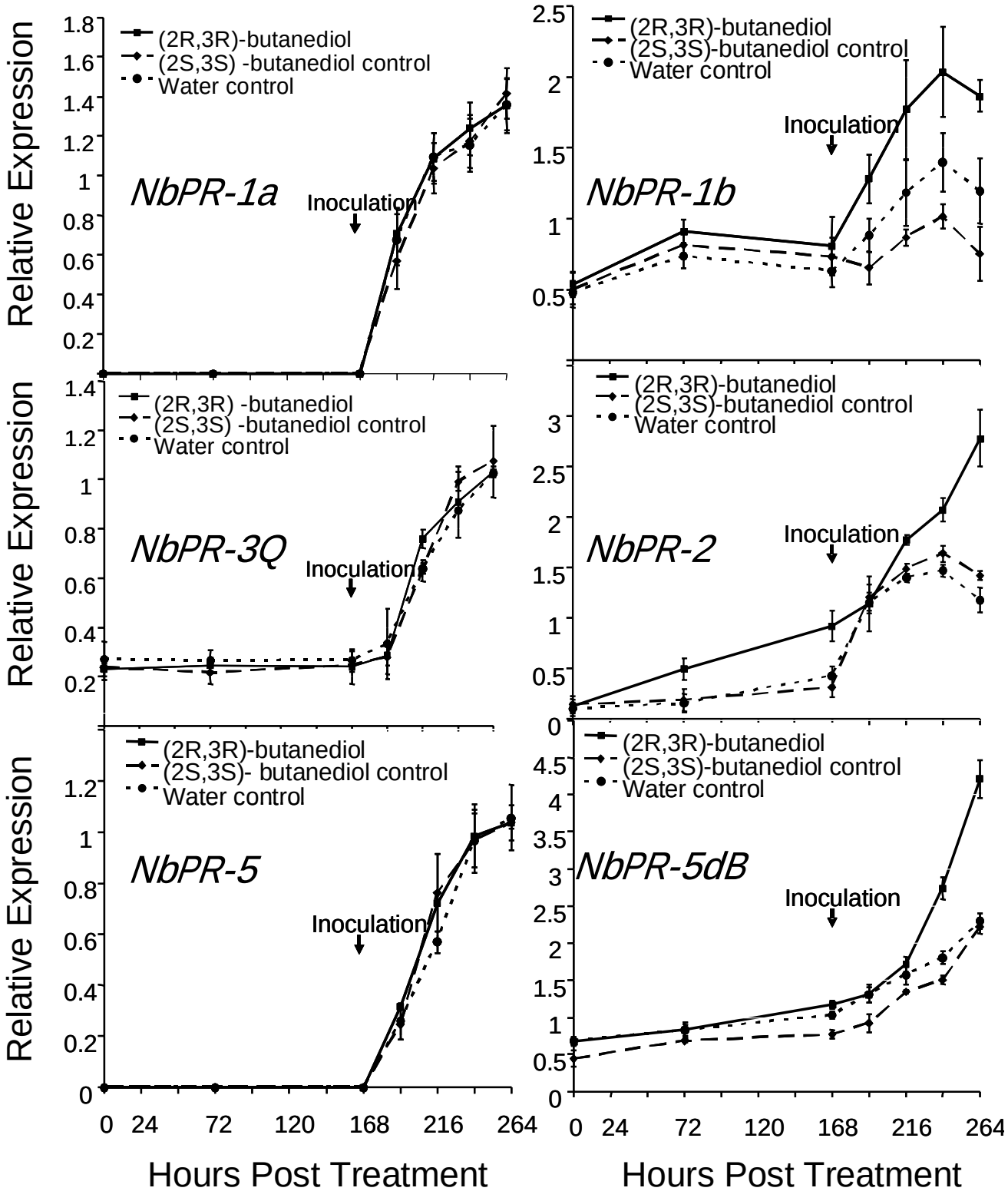
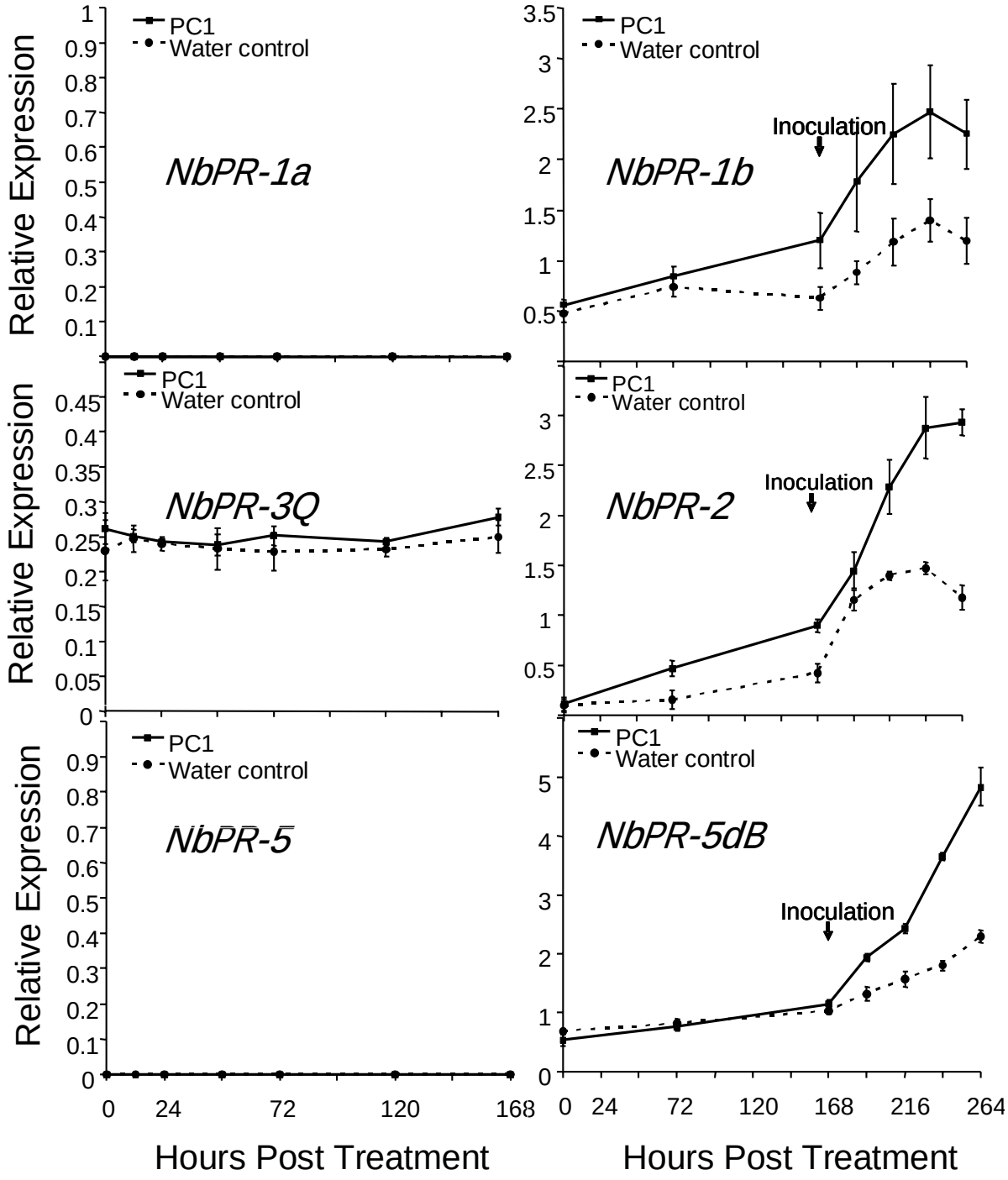

Relative Expression
(2R,3R)-butanediol
(2S,3S) -butanediol control
Water control
NbPR-1a
Inoculation
(2S,3S)-butanediol control
NbPR-1b
(2R,3R) -butanediol
NbPR-3Q
NbPR-2
(2S,3S)- butanediol control
NbPR-5
NbPR-5dB
0 24 72 120 168 216 264
Hours Post Treatment

Fig 3


PC1
Water control
NbPR-1a
NbPR-1b
NbPR-3Q
NbPR-2
NbPR-5
NbPR-5dB
Inoculation
Relative Expression
Hours Post Treatment
0
24
72
120
168
216
264