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Original Article

Induction of pathogenesis-related gene 1 (*PR-1*) by acibenzolar-s-methyl application in pineapple and its effect on reniform nematodes (*Rotylenchulus reniformis*)

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ABSTRACT

The induction of systemic acquired resistance (SAR) in pineapples (*Ananas comosus)* was studied as shown by the up-regulation of the *PR-1* gene (the SAR marker) and examination of the SAR effect on the reniform nematode, *Rotylenchulus reniformis*. Real-time polymerase chain reaction assay was performed using degenerate primers designed from the *PR-1* genes of several monocotyledonous (monocots) and dicotyledonous (dicots) plants. A 266 bp cDNA band was evident only in plants treated with the SAR inducer, acibenzolar-s-methyl. This 266 bp cDNA was sequenced and found to be highly homologous to a number of *PR-1* genes from monocots. In addition, the amino acid sequence deduced from the 266 bp cDNA showed a high identity to PR-1 proteins from both monocots and dicots. Therefore, it was highly likely that this cloned fragment was part of the *A. comosus PR-1* gene, indicating that *A. comosus* has an SAR pathway. The time course of *PR-1* expression was studied. The results showed that *PR-1* induction was initiated as early as 1 d after acibenzolar application and continued through 3 wk thereafter. The effect of SAR on the nematodes, *R. reniformis*, in pineapples was also elucidated. The results showed that the reproduction of nematodes on the pineapples treated with 100 mg/L or 200 mg/L was 55% lower than that on pineapples treated with 0 mg/L or 50 mg/L. Nematode reproduction on pineapples treated with the same concentration but inoculated at different times was not significantly different ($p > 0.05$).

Introduction

Systemic acquired resistance (SAR) can be triggered by a necrotic lesion either formed as part of a hypersensitivity reaction in an incompatible reaction or as a symptom of disease in a compatible interaction (Ryals et al., 1996). SAR can also be induced by the exogenous application of chemical inducers, such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), or acibenzolar-s-methyl (Ward et al., 1991; Uknes et al., 1992; Vernooij et al., 1995; Friedrich et al., 1996; Lawton et al., 1996; Fu and Dong, 2013).

One of the best described characteristics of SAR is the accumulation of pathogenesis-related (PR) proteins in the induced plants—PR proteins normally accumulate at a faster rate in incompatible than in compatible reactions (Hammond-Kosack and Jones, 1996). In plants like tobacco and *Arabidopsis*, the establishment of SAR is tightly correlated with the expression of PR proteins (Ward et al., 1991; Uknes et al., 1992; Ryals et al., 1996; Hammerschmidt, 2007).

The identity and relative expression levels of *PR* genes vary between plant species (Ryals et al., 1996). In tobacco, at least nine families of *PR* genes (*PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, acidic and basic isoforms of class III chitinase, an extracellular ß-1, 3-glucanase [*PR-Q'*] and the basic isoform of *PR-1*) were expressed during the onset of SAR (Ward et al., 1991). In *Arabidopsis*, however, only three *PR* genes (*PR-1*, *PR-2* and *PR-5*) were induced (Uknes et al., 1992). In both cases, the acidic *PR-1* was the predominant *PR* gene. Although the pattern of gene expression differs among plant species, the induction of *PR-2*, *PR-5* and especially *PR-1* by pathogens and chemicals occurs in most dicotyledonous plants and consequently, these genes can serve as SAR markers (Ward et al., 1991; Uknes et al., 1992; Friedrich et al., 1996; Lawton et al., 1996).

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In monocotyledonous plants, several homologs of the dicotyledonous *PR* genes have been identified. In maize and barley, *PR-1* and *PR-5* were induced in both incompatible and compatible interactions (Stevens et al., 1996; Morris et al., 1998). The expression of genes encoding acidic and basic *PR-1* was evident in barley treated with INA and in maize treated with acibenzolar (Muradov et al., 1993; Kogel et al., 1994; Morris et al., 1998).

A study by Sudarshan et al. (2011) demonstrated that the application of benzothiadiazole (acibenzolar-s-methyl) or infection of cotton by *Meloidogyne incognita* or *Rotylenchulus reniformis* enhanced the activity of the enzymes involved in SAR such as P-peroxidase, G-peroxidase and catalase. Melillo et al. (2014) illustrated that treatment of tomato with benzothiadiazole increased the accumulation of lignin and H_2O_2 and also induced the expression of two tomato genes—*Tap1* and *Tap2*—encoding anionic peroxidases. Molinari (2015) concluded that SA, INA and benzothiadiazole induced SAR on solanaceous crops and could be included in integrated pest management programs for nematode management.

Chinnasri et al. (2006) in a study with pineapple, reported that the application of acibenzolar-s-methyl at 100 mg/L of water to pineapple reduced reproduction of *R. reniformis* and *Meloidogyne javanica*. SAR was hypothesized to be induced in pineapple treated with acibenzolar. To verify that SAR was activated by acibenzolar application, the induction of the SAR marker gene *PR-1* in pineapple was determined in the current study.

Materials and methods

RNA extraction

Six crowns of pineapple (*Ananas comosus* cv. Smooth Cayenne) were planted in 15-cm-diameter clay pots containing sterile, sandy loam soil and placed in a greenhouse. One month later, three plants were foliar sprayed to runoff with water (control) and another three plants with a solution of 100 mg acibenzolar-s-methyl/L of water, provided under the commercial name of Actigard 50 WG® (Syngenta; Greensboro, NC, USA). To prevent runoff into the soil, the surface of the soil was covered with a plastic sheet while the foliage was sprayed.

Two days after treatment, plants were removed from the pots and the soil gently washed from the roots. The roots were cut off from the crowns at their bases and placed on ice. The roots were immediately processed to extract RNA.

RNA was extracted from roots using the protocol developed by Hayden and Christopher (2004). Contaminating DNA was removed from the RNA samples by treating with (RNase-free) DNase. RNA was quantified by measuring the OD_{260} (1 optical density = 40 μg/mL). Its purity was calculated by dividing OD_{260} by OD_{280}. RNA intactness was analyzed using electrophoresis on 1.0% formaldehyde/1.2% agarose gels [0.2 M N-morpholino propanesulfonic acid, 50 mM sodium acetate, and 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0].

Primers for polymerase chain reaction

Sense [5′-GCC CGC GCC GCC GTC GGC GTG-3′] and antisense [5′-CCA CAC CAC CTG CGT GTA GTG-3′] degenerate primers were designed from the consensus I and consensus II of the rice basic *PR-1* gene. For the pineapple *ubiquitin* gene (accession numbers AY098513 and AY098526), a pair of primers [sense 5′-ACC AGC AGC GGT TGA TCT TCG CCG-3′ antisense 5′-AGT GCG GCC GTC CTC CAA CTG C-3′] was derived. The pineapple *ubiquitin*-specific primers were designed to have a polymerase chain reaction (PCR) product size (266 bp) and annealing temperature (65 °C) similar to the *PR-1* specific primers to avoid differences in the PCR kinetics.

Amplification of a PR-1-related polymerase chain reaction product from pineapple by reverse transcription-polymerase chain reaction

The first-strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega; Madison, WI, USA). For primer annealing, a mixture of 1 μL oligo dT (100 ng/μL), 10 μg total RNA and 19 μL water was incubated at 65 °C for 10 min. To start first strand cDNA synthesis, 12 μL 5X M-MLV buffer, 2 μL 10 mM dNTPs, 1 μL RNasin, 1 μL M-MLV Reverse Transcriptase and 14 μL water were added to each tube. The reaction was incubated at 37 °C for 1 h followed at 42 °C for 30 min and then kept on ice.

A traditional PCR reaction to amplify the first strand cDNA was followed. The components of the PCR were: 5 μL 10X buffer, 3 μL 25 mM $MgCl_2$, 10 μL 1 mM dNTPs, 1 μL 100 ng/μL sense *PR-1* specific-primer, 1 μL 100 ng/μL antisense *PR-1* specific primer, 5 μL cDNA template, 0.5 μL Taq DNA polymerase (Promega; Madison, WI, USA) at 5 units/μL and 24.5 μL water. The PCR was performed for 40 cycles. Each cycle consisted of 30 s at 94 °C for denaturing, 30 s at 65 °C for annealing, and 1 min at 72 °C for extension. PCR products were fractionated on 2.0% agarose gel in 1X Tris Borate EDTA (TBE) buffer.

Cloning of the polymerase chain reaction product

The DNA fragment amplified in PCR was purified and cloned using a GFX kit (Amersham; Piscataway, NJ, USA) and a TOPO TA cloning kit (Invitrogen; Waltham, MA, USA, respectively). Four μL of purified PCR product were added along with 1 μL salt solution and 1 μL pCR®4-TOPO plasmid vector to a 0.2 mL PCR tube. The tube was incubated at 24 °C for 30 min and the mixture was then transformed into competent *Escherichia coli*. Competent *E. coli* cells (DH5α strain) were prepared using Inoue's method (Inoue et al., 1990) and kept frozen at −80 °C. *E. coli* cells were transferred from −80 °C to ice for thawing and the ligation mix (6 μL) was added. After 40 min of incubation on ice, cells were heat shocked at 42 °C for 30 s and synchronized on ice for 10 min. Then, 1 mL Luria-Bertani (LB) media without antibiotics was added to the tube and incubated on a shaker at 37 °C for 40–60 min. Cells were then collected by centrifugation (5000× *g* for 30 s). The supernatant was removed and the *E. coli* cells resuspended in the remaining media (approximately 100 μL). Cells were then spread on LB media containing ampicillin (100 μg/mL), X-Gal (32 μg/mL) and 16 μM isopropyl-β-D-thiogalactopyranoside and incubated overnight at 37 °C. White colonies were then transferred to another LB Amp^{100} media plate using colony streaking and incubated at 37 °C for 18 h. For plasmid DNA isolation, single colonies were picked from the plate and grown overnight in LB Amp^{100} at 37 °C.

Purification and digestion of plasmid DNA

To isolate plasmid DNA from *E. coli*, the plasmid miniprep (Qiagen; Valencia, CA, USA) kit was used. A 1.5 mL sample of the overnight *E. coli* culture (OD ≥ 1) was pelleted in a 1.5 mL Eppendorf tube by centrifugation at 16,000× *g* for 30 s, resuspended sequentially in 250 μL Buffer P1, 250 μv Buffer P2, and 350 μL Buffer N3 and mixed thoroughly after each step. Centrifugation at 16,000× *g* was conducted for 10 min and the supernatant was applied to a QIAprep column. After centrifugation at 16,000× *g* for 30–60 s, the column was washed by adding 0.5 mL Buffer PB and 750 μL buffer PE. An additional 1 min of centrifugation at 16,000× *g* was conducted to remove any residual wash buffer. The QIAprep column was placed in a clean 1.5 mL microcentrifuge tube and 50 μL

Buffer EB (10 mM Tris-Cl, pH 8.5) added to the center of the column. The column was allowed to stand for 1 min and centrifuged at 16,000× *g* for 1 min. The flow-through contained the plasmid DNA. Concentration was measured at OD_{260}. To determine if the insert was present in the plasmid vector, 500 ng plasmid DNA was digested with *Eco*RI by adding 1 μL 10X buffer (Promega; Madison, WI, USA) and 0.5 μL *Eco*RI enzyme (20 U/μL) in 10 μL of final volume. The reaction was incubated at 37 °C for 1 h. Digested DNA fragments were separated on 2% agarose gel in 1X TBE buffer.

Preparation of plasmid DNA for sequencing

An amount of 1 μg of plasmid DNA was thoroughly mixed with 50 μL 95% ethyl alcohol and 2 μL 3.0 M NaOAc (pH 5.2). The reaction was incubated at −70 °C for 10 min and centrifuged at 16,000× *g* for 5 min. The pellet was washed with 70% ethanol, centrifuged at 16,000× *g* for 2 min and dried. Then, 11 μL of ultrapure deionized water were added to the tube. The plasmid vector was transferred to a sequencing tube and 1 μL 100 ng/μL M13 reverse primer was added. The sample was then sent to the University of Hawaii Biotech Core Facility (Honolulu, HI, USA) for sequencing.

Determining the level of PR-1 gene expression

Four 1-month-old pineapples were sprayed with a solution of acibenzolar at a concentration of 100 mg/L. Four 1-month-old pineapples treated with water served as controls. Then, 1 d, 7 d, 14 d and 21 d after the acibenzolar or water application, RNA samples were extracted and the intactness and quantity were determined as previously described.

Reverse transcriptase and normal PCR reactions were conducted for different treatments (water or acibenzolar application) and collection times (days 1, 7, 14, or 21 post application) with the *PR-1* and *ubiquitin* primer sets. Constitutive expression of the nonacibenzolar-induced gene *ubiquitin* of pineapple served as an internal standard.

PCR reactions were performed for 25 cycles, 30 cycles, 35 cycles or 40 cycles. Each cycle consisted of 30 s at 94 °C for denaturing, 30 s at 65 °C for annealing and 1 min at 72 °C for extension. PCR products were fractionated on 2.0% agarose gel in 1X Tris Borate EDTA (TBE) buffer.

The study was repeated once with a new set of eight 1-month-old plants.

Effects of systemic acquired resistance on reniform nematodes, R. reniformis

R. reniformis nematodes were reared on *Vigna unguiculata* 'California Black Eye' in the greenhouse. After 6 wk, nematode eggs were extracted using 1% NaOCl (Byrd et al., 1972).

Crowns of pineapples (*A. comosus* cv. Smooth Cayenne) were planted in 15-cm-diameter clay pots. One month later, pineapple plants were foliar applied with a solution of 0 mg acibenzolar/L of water, 50 mg acibenzolar/L of water, 100 mg acibenzolar/L of water or 200 mg acibenzolar/L of water (10 mL of the solution per plant). While acibenzolar was applied, the soil surface was covered with a plastic sheet to prevent runoff. Then, 1 d, 7 d, 14 d or 21 d after the acibenzolar application, 10,000 eggs of *R. reniformis* were inoculated onto the soil around each pineapple plant. One month after inoculation at each time point, nematode reproduction was assessed. The pineapple plants were uprooted, gently watered to remove soil and cut at their bases. Nematode eggs were extracted from the pineapple roots by shaking the roots in 1% NaOCl (Byrd et al., 1972). The nematode vermiform stages were extracted from 250 cm^3 soil using elutriation and centrifugation (Byrd et al., 1976; Jenkins, 1964).

The experiment was a 4 × 4 factorial with four replications (plants) and arranged in a completely randomized design. The experiment was repeated once. ANOVA was used to determine the effect of acibenzolar and inoculation time on nematode reproduction. Tukey's studentized range (HSD) test was used to determine differences among acibenzolar concentrations using the SAS statistical software (SAS Institute; Cary, NC, USA).

Results

Amplification of a PR-1-related polymerase chain reaction product from pineapple by reverse transcription-polymerase chain reaction

PR-1 specific degenerated primers were designed by determining the alignment of the PR-1 amino acid sequences from several monocotyledonous and dicotyledonous plants (Agrawal et al., 2000). In consensus I and II of the rice basic PR-1 protein, amino acid sequences of ARAAVGV and HYTQVVW, respectively, showed the highest homology to the rice acidic, wheat, maize (acidic and basic), tobacco and *Arabidopsis* PR-1 protein. To search for nucleotide sequences, the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used and the amino acid sequences of ARAAVGV and HYTQVVW corresponded to 5′-GCC CGC GCC GCC GTC GGC GTG-3′ and 5′-CAC TAC ACG CAG GTG GTG TGG-3′, respectively. These two nucleotide sequences also showed high similarity to the *PR-1* genes from several plants. Therefore, they were used to design *PR-1*-specific sense and antisense degenerate primers for the RT-PCR reaction (see sequences in the Materials and methods).

Following the RT-PCR reaction, a cDNA fragment of between 271 and 281 bp was present only in pineapple treated with acibenzolar-s-methyl as expected (Fig. 1). Since there was no PCR product in the control, it was assumed that this PCR product was derived from the pineapple *PR-1* gene. For cloning, the strongest PCR product (number 4) was isolated.

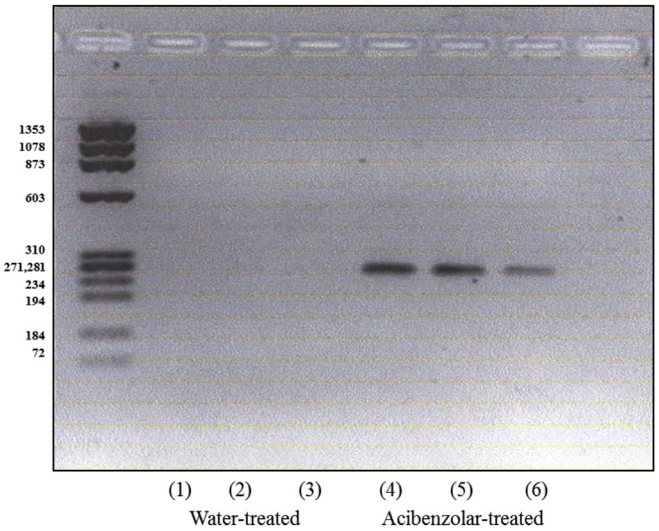

Fig. 1. Detection of *PR-1* gene expression in the roots of pineapples by reverse transcriptase-polymerase chain reaction. (1) (2) (3) represent three individual plants treated with water (control). (4) (5) (6) constitute three individual plants treated with acibenzolar-s-methyl. The sizes of the molecular weight marker (phiX174/HaeIII) are indicated on the left column. PCR was performed for 40 cycles.

Sequence a of the cloned cDNA

The sequence of the cloned cDNA is shown in Fig. 2. The cloned cDNA fragment was 266 bp long. The 5′ and 3′ end of the fragment corresponded to the primers used for amplification. At the 5′ end, the first 21 bases were identical to the *PR-1* antisense primer. At the 3′ end, the first 21 bases were complementary to the *PR-1* sense primer.

The BLASTN program (National Center for Biotechnology Information; Bethesda, MD, USA) was used to compare the nucleotide sequence with the database. The cloned fragment showed significant homology to other *PR-1* genes. It had high homology to the *Hordeum vulgare Bpr* 1-1 gene for the type 1 pathogenesis-related protein. The stretch of nucleotides from 1 to 165 of the cloned fragment was 79% identical to that from 909 to 745 of the *H. vulgare Bpr 1-1* gene. Also, high similarity was shown to the *H. vulgare HvPR-1a* gene. The cDNA fragment also shared homology to the *PR-1* genes from several plants such as *Zea mays*, *Triticum aestivum*, *Oryza sativa*, and *Linum usitatissimum*. The comparison also found homology with *O. sativa* (japonica cultivar-group) genomic DNA (chromosomes 1, 2, 7 and 10), and *Arabidopsis thaliana* DNA chromosome 4. In addition, some sections of genes from bacteria such as *Chromobacterium violaceum* 12,472 and *Pseudomonas aeruginosa* PAO1 were found to be homologous to parts of the 266 cDNA fragment. However, their percentage homology was low.

The amino acid sequence was analyzed using BLASTX. The cloned 266 bp cDNA fragment was translated into an 88 amino acid polypeptide and the frame-3 was the most possible translation frame. The translated amino acid sequence was: WVVQTYHGCV KGSMCTNSRY DYYQKEAVWL EVADSATWTK GSGWFLNEGY PGGSHILKCD GRRQNAYNQA YAELNNDWSL PGRRRRRP.

The translated polypeptide had the highest identity to the PR-1 protein from *Z. mays* (73%), followed by that from *H. vulgare* (70%), *T. aestivum* (69%), *O. sativa* (67%), *Nicotiana tabacum* (67%), *Lycopersicon esculentum* (63%), *A. thaliana* (61%) and *Cucumis sativus* (58%).

Determining the level of PR-1 gene expression with reverse transcription-polymerase chain reaction

Expression of *ubiquitin,* the internal standard, was similar in water and acibenzolar-treated pineapple (Fig. 3), indicating no major cellular change following the application of acibenzolar. Even though some unspecific products appeared, these occurred in a similar pattern in both the water-treated and acibenzolar-treated pineapple samples. The intensity of the *ubiquitin* bands was similar in all cycles, indicative of being a constitutive and abundantly-expressed gene in pineapple.

Expression of the *PR-1* gene was not detected at 25 PCR cycles or 30 PCR cycles (Fig. 3). At 35 cycles, however, the *PR-1* gene was induced as early as 1 d post acibenzolar treatment and expression continued through 21 d after the acibenzolar application. However, the *PR-1* gene expression was also detected in the water-treated pineapple at 14 d and 21 d thereafter. This supported the assumption that the *PR-1* gene in pineapple is also responsive to stress. A similar pattern of expression was evident in the 40 PCR cycle samples. *PR-1* was induced 1 d after the acibenzolar treatment and remained activated at 21 d. *PR-1* in the water-treated pineapple was detected only in the latter stages (days 14 and 21).

The results of the repeat study were similar to the first experiment (Fig. 4). The intensity of the *ubiquitin*-derived PCR product bands of the water-treated pineapple was similar to that of the acibenzolar-treated pineapple (Fig. 4). In addition, increasing the number of PCR cycles did not heighten the intensity of the *ubiquitin* PCR product bands. This was consistent with the result where foliar application of acibenzolar did not render any major changes to pineapple. The *PR-1* gene was activated differentially between the water-treated and acibenzolar-treated pineapple (Fig. 4). No *PR-1* gene induction was detected in the water-treated plants throughout the 21 d of the study. However, similar to the first experiment, the *PR-1* specific PCR band appeared in the acibenzolar-treated samples after 1 d and remained activated for 21 d.

Effects of systemic acquired resistance on reniform nematodes, R. reniformis

R. reniformis inoculated at different time points did not produce different reproduction rates ($p > 0.05$) as shown in Fig. 5. In addition, there was no interaction between inoculation times and acibenzolar concentrations ($p > 0.05$). However, nematode reproduction was highly significantly different on pineapples applied with acibenzolar at different concentrations ($p < 0.01$) as shown ion Fig. 5.

Nematode reproduction on pineapples was reduced by approximately 50% when the plants were applied with acibenzolar at a concentration of 100 mg/L or 200 mg/L of water ($p < 0.01$) as shown in Fig. 5. At these concentrations, whether nematodes were inoculated 1d, 7 d, 14 d or 21 d post acibenzolar treatment, the nematode reproduction was the same ($p > 0.05$) as shown in Fig. 5.

Discussion

Most mature PR-1 proteins have 135 amino acids encoded by about 405 base pairs (Van Loon and Van Strien, 1999; Van Loon et al., 2006). The cloned fragment had 266 bases, indicating it was more than half of an average full-sized *PR-1* gene. It is likely that this fragment was derived from a *PR-1* gene or a *PR-1* gene family member of *A. comosus*. The BLASTN nucleotide comparison showed most similarity to *PR-1* genes from a number of

```
1    CCACACCACC  TGCGTGTAGT  GCCCGCAAAC  TTTCCCGGAC  ATGCACGTGT
51   TGCTGCGGTA  GTCGTAGTAT  TGCTTCTCCG  CCACCCACAA  CTCCACTGCG
101  TCGGAGGCCG  TCCATGTTTT  GCCAGAGCCC  CAGAAGAGGT  TCTCGCCGTA
151  GGGCCCACCA  GAGTGGATGA  GTTTGCAGTC  TCCGCGCCTT  TGATTGGCAT
201  AGTTTTGCGC  GTACGCCTCC  AGGTTGTTGT  CCCAGCTCAA  AGGGCCACGC
251  CGACGGCGGC  GCGGGC
```

Fig. 2. Nucleotide sequence of cloned 266 bp cDNA fragment (in bold) amplified from *Ananas comosus* roots treated with acibenzolar-s-methyl. Nucleotides in regular style represent those of the pCR®4-TOPO vector. Bases underlined are *PR-1* primer antisense and *PR-1* primer sense.

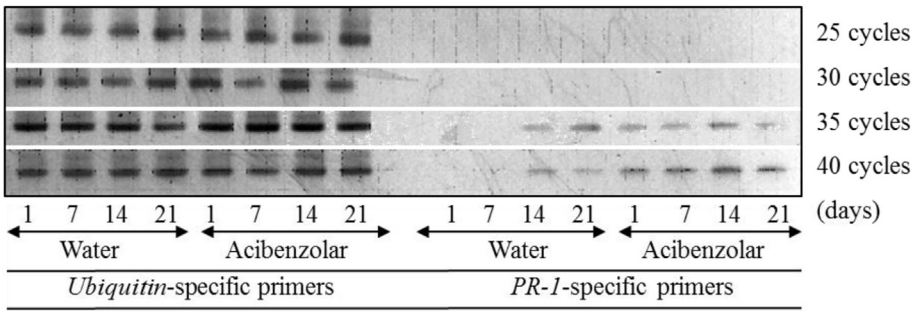

Fig. 3. Time course of *PR-1* gene expression in pineapples following treatment with water or 100 mg/L acibenzolar (1 d, 7 d, 14 d, or 21 d post treatment). Polymerase chain reaction (PCR) was performed using *PR-1*-specific primers for different numbers of cycles. PCR using *ubiquitin*-specific primers served as the internal standard.

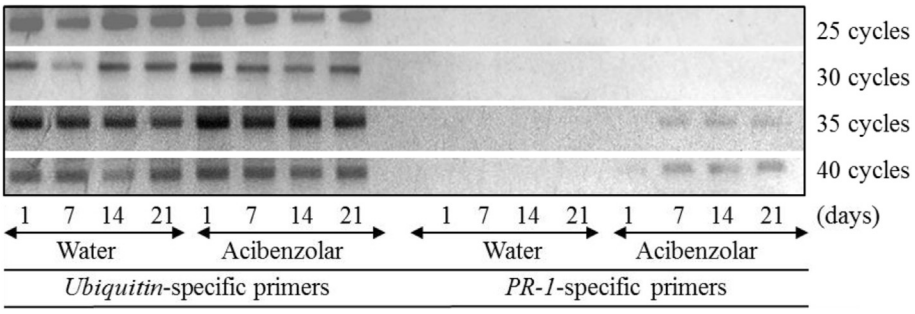

Fig. 4. A repeat of time course of *PR-1* gene expression in pineapples following treatment with water or 100 mg/L acibenzolar (1 d, 7 d, 14 d or 21 d post treatment). Polymerase chain reaction (PCR) was performed using *PR-1*-specific primers for different numbers of cycles. PCR using *ubiquitin*-specific primers served as the internal standard.

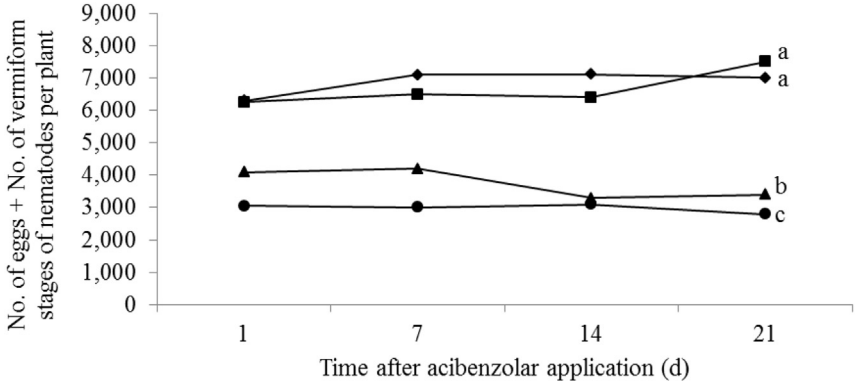

Fig. 5. Effects of systemic acquired resistance induced by acibenzolar at 0 mg/L (◆), 50 mg/L (■), 100 mg/L (▲) and 200 mg/L (●) on the reniform nematode, *Rotylenchulus reniformis*. Pineapples were challenged with *R. reniformis* 1 d, 7 d, 14 d or 21 d after acibenzolar application. Data are means of eight replications (plants) from two experiments, and those with the same lowercase letters are not different ($p > 0.05$), based on Tukey's studentized range (HSD) test.

monocotyledonous plants. Homology was low with accessions other than *PR-1* genes.

The homology with proteins other than PR-1 was not found when the amino acid sequence deduced from the cloned fragment was evaluated. In addition, this amino acid sequence showed high similarity to the PR-1 proteins from dicotyledonous plants such as tobacco, *Arabidopsis*, cucumber and tomato. These data suggested that this 266 bp cDNA fragment is part of a pineapple *PR-1* gene. In addition, the expression of this cDNA fragment was in response to the application of acibenzolar-s-methyl—a known SAR inducer. To ensure that this was the pineapple PR-1 protein, the full-length cDNA of the 266 bp fragment needs to be cloned from pineapple.

It is likely that the pineapple *PR-1* gene is also responsive to some factors other than acibenzolar treatment. Environmental stress has been shown to regulate *PR* gene expression as Van Loon and Van Strien (1999) showed that some *PR* genes were responsive to both biotic and abiotic factors. In tobacco and tomato, *PR-5* was induced in response to osmotic stress (Singh et al., 1987). Agrawal et al. (2000) found that the acidic form of the rice *PR1* gene was not only responsive to jasmonic acid, but also was cut-sensitive. Radovich et al. (2005) found the level of proteinase inhibitor—perhaps a PR-6 analog—in the roots of pineapple increased with plant age, with proteinase inhibitor reaching a maximum 6 mth after planting, followed by a slight decrease and subsequent leveling off. In addition, the level of proteinase inhibitor seemed to be responsive to temperature when data collected in fall and spring were compared.

PR genes have also been reported to have a developmental role. Neale et al. (1990) found that several *PR* genes were induced upon the transition of plants from vegetative growth to flowering.

Brederode et al. (1991) and Stintzi et al. (1991) demonstrated that tobacco "basic PR-5" was not present in young leaves but was produced in leaves upon their maturation and senescence.

Further study is needed to elucidate whether the *PR-1* gene in pineapple is a good marker for SAR induction. In this study, the *PR-1* gene may have been expressed in the water-treated pineapple at a level lower than that in acibenzolar-treated plants. To detect the differences and gain an insight into the more detailed characteristics of *PR-1* gene expression, real-time reverse-transcription polymerase chain reaction may be a helpful method to answer these questions.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Agrawal, G.K., Jwa, N.S., Rakwal, R., 2000. A novel rice (*Oryza sativa* L.) acidic *PR1* gene highly responsive to cut, phytohormones, and protein phosphatase inhibitors. Biochem. Biophys. Res. Commun. 274, 157–165.

Brederode, F.T., Linthorst, H.J.M., Bol, J.F., 1991. Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol. Biol. 17, 1117–1125.

Byrd Jr., D.W., Ferris, H., Nusbaum, C.J., 1972. A method for estimating numbers of eggs of *Meloidogyne* spp. in soil. J. Nematol. 4, 266–269.

Byrd Jr., D.W., Barker, K.R., Ferris, H., Nusbaum, C.J., Griffin, W.E., Small, R.H., Stone, C.A., 1976. Two semi-automatic elutriators for extracting nematodes and certain fungi from soil. J. Nematol. 8, 206–212.

Chinnasri, B., Sipes, B.S., Schmitt, D.P., 2006. Effects of inducers of systemic acquired resistance on reproduction of *Meloidogyne javanica* and *Rotylenchulus reniformis* in pineapple. J. Nematol. 38, 319–325.

Friedrich, L., Lawton, K., Ruess, W., et al., 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J. 10, 61–70.

Fu, Z.Q., Dong, X., 2013. Systemic acquired resistance: turning local infection into global defense. Annu. Rev. Plant Biol. 64, 839–863.

Hammerschmidt, R., 2007. Introduction: definition and some history, pp. 1–8. In: Walters, D., Newton, A., Lyon, G. (Eds.), Induced Resistance for Plant Defense: a Sustainable Approach to Crop Protection. Blackwell Publishing, Oxford, UK.

Hammond-Kosack, K.E., Jones, J.D.G., 1996. Resistance gene-dependent plant defense responses. Plant Cell 8, 1773–1791.

Hayden, D.M., Christopher, D.A., 2004. Characterization of senescence-associated gene expression and senescence-dependent and −independent cysteine proteases differing in microsomal processing in *Anthurium*. Plant Sci. 166, 779–790.

Inoue, H., Nojima, H., Okayama, H., 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene 96, 23–28.

Jenkins, W.R., 1964. A rapid centrifugal-flotation technique for separating nematodes from soil. Plant Dis. Rep. 48, 692.

Kogel, K.H., Beckhove, U., Dreschers, J., Münch, S., Rommé, Y., 1994. Acquired resistance in barley. Plant Physiol. 106, 1269–1277.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., Ryals, J., 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J. 10, 71–82.

Melillo, M.T., Leometti, P., Veronico, P., 2014. Benzothiadiazole effect in the compatible tomato-*Meloidogyne incognita* interaction: changes in giant cell development and priming of two root anionic peroxidases. Planta 240, 841–854.

Molinari, S., 2015. Systemic acquired resistance activation in solanaceous crops as a management strategy against root-knot nematodes. Pest Manag. Sci. 72, 888–896.

Morris, S.W., Vernooij, B., Titatarn, S., et al., 1998. Induced resistance responses in maize. Mol. Plant Microbe Interact. 11, 643–658.

Muradov, A., Petrasovits, L., Davidson, A., Scott, K.J., 1993. A cDNA clone for pathogenesis-related protein 1 from barley. Plant Mol. Biol. 23, 439–442.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.J., Dennis, E.S., 1990. Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2, 673–684.

Radovich, C., Paull, R., Sipes, B.S., 2005. Protease inhibitors and reproduction of *Rotylenchulus reniformis* in pineapple. Acta Hortic. 666, 223–228.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., Hunt, M.D., 1996. Systemic acquired resistance. Plant Cell 8, 1809–1819.

Singh, N.K., Bracker, C.A., Hasegawa, P.M., et al., 1987. Characterization of osmotin. A thaumatin-like protein associated with osmotic adaptations in plant cells. Plant Physiol. 85, 529–536.

Stevens, C., Titarenko, E., Hargreaves, J.A., Gurr, S.J., 1996. Defence-related gene activation during an incompatible interaction between *Stagonospora (Septoria) nodorum* and barley (*Hordeum vulgare* L.) coleoptile cells. Plant Mol. Biol. 31, 741–749.

Stintzi, A., Heitz, T., Kauffmann, S., Legrand, M., Fritig, B., 1991. Identification of a basic pathogenesis-related, thaumatin-like protein of virus-infected tobacco as osmotin. Physiol. Mol. Plant Pathol. 38, 137–146.

Sudarshan, K.A., Davis, R.F., Stevenson, K.L., Timper, P., Ji, P., 2011. Influence of infection of cotton by *Rotylenchulus reniformis* and *Meloidogyne incognita* on the reproduction of enzymes involved in systemic acquired resistance. J. Nematol. 43, 152–159.

Uknes, S., Mauch-Mani, B., Moyer, M., et al., 1992. Acquired resistance in *Arabidopsis*. Plant Cell 4, 645–656.

Van Loon, L.C., Van Strien, E.A., 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55, 85–97.

Van Loon, L.C., Rep, M., Pieterse, C.M.J., 2006. Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44, 135–162.

Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H., Ryals, J., 1995. 2,6-dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. Mol. Plant Microbe Interact. 8, 228–234.

Ward, E.R., Uknes, S.J., Williams, S.C., et al., 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3, 1085–1094.