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# The folate precursor *para*-aminobenzoic acid elicits induced resistance against *Cucumber mosaic virus* and *Xanthomonas axonopodis*

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• *Background and Aims* The use of vitamins including vitamin $B_1$, $B_2$ and $K_3$ for the induction of systemic acquired resistance (SAR) to protect crops against plant pathogens has been evaluated previously. The use of vitamins is beneficial because it is cost effective and safe for the environment. The use of folate precursors, including *ortho*-aminobenzoic acid, to induce SAR against a soft-rot pathogen in tobacco has been reported previously.
• *Methods* In the present study, *para*-aminobenzoic acid (PABA, also referred to as vitamin $B_x$) was selected owing to its effect on the induction of SAR against *Xanthomonas axonopodis* pv. *vesicatoria* in pepper plants through greenhouse screening.
• *Key Results* Dipping of pepper seedlings in a 1 mM PABA solution in field trials induced SAR against artificially infiltrated *X. axonopodis* pv. *vesicatoria* and naturally occurring cucumber mosaic virus. Expression of the *Capsicum annuum pathogenesis-related 4* gene was primed in response to pathogen infection as assessed by quantitative real-time PCR. The accumulation of cucumber mosaic virus RNA was reduced in PABA-treated pepper plants at 40 and 105 d post-treatment. Unexpectedly, fruit yield was increased in PABA-treated plants, indicating that PABA-mediated SAR successfully protected pepper plants from infection by bacterial and viral pathogens without significant fitness allocation costs.
• *Conclusions* The present study is the first to demonstrate the effective elicitation of SAR by a folate precursor under field conditions.

**Key words:** Systemic acquired resistance, defence priming, induced systemic resistance, plant growth-promoting rhizobacteria, PGPR, folate, *para*-aminobenzoic acid, PABA, vitamin $B_x$.

## INTRODUCTION

Systemic acquired resistance (SAR) is a plant defence response to pathogen attack. SAR is induced by various biotic and abiotic factors including necrotizing pathogens and chemicals, and was first reported as early as 1966 by Ross, who identified plant virus-elicited SAR (Ross, 1961). Because the use of necrotizing pathogens or their metabolites for the induction of SAR in field trials is associated with adverse effects, research has focused on the synthesis of SAR-inducing microbial metabolite and chemicals during the last decades (Lyon, 2007). Plants recognize specific molecules as 'non-self' and several microbial-derived compounds such as antibiotics, chitin, ergosterol, glucans, lipopolysaccharides, proteins, peptides, salicylic acid (SA), sphingolipids and volatile organic compounds elicit plant immunity against diverse pathogens (Lyon, 2007). However, recently, a defence mechanism similar to SAR was described in plants and named induced systemic resistance (ISR) and microbe-associated molecular pattern-triggered immunity, which is activated by beneficial root-associated bacteria referred to as plant growth-promoting rhizobacteria (PGPR; see Table 1 for a list of abbreviations) and multiple microbe-associated molecular patterns including bacterial flagellin, elongation factor Tu and fungal chitin (Zipfel, 2009; Kloepper *et al.*, 2004). Furthermore, many plant metabolites and hormones, including brassinolide, jasmonate, oligogalacturonides, oxalate, spermine, ethylene and volatile organic compounds, induce SAR (Hammerschmidt, 2009). Benzothiadizole (BTH), which was commercialized by Novartis Co. (now changed to Syngenta) as Actigard® in the USA and BION® in Europe, is the first SAR-inducing commercialized chemical that is effective against a broad spectrum of pathogens, including oomycetes in wheat and tobacco and whitefly in tomato, and is effective for up to 70 d (Tally *et al.*, 1999). BTH application increases endogenous SA levels and can activate SA-dependent signalling pathways in plants. BTH induces SAR in SA-hydrolysing *NahG* transgenic plants, indicating that it can induce resistance in the absence of SA-signalling (Molina *et al.*, 1998). This feature of SAR, which is effective against a wide range of pathogens and herbivores, is the aim of most disease management strategies (Hammerschmidt, 2009).

Although chemical SAR inducers have been intensively studied for potential agrochemical development, the chemical inducers, BTH and DL-3-aminobutyric acid (BABA), are reported to have a negative effect on plant growth (Heil *et al.*, 2000; Van Hulten *et al.*, 2006). This phenomenon is known as 'allocation fitness cost' or 'trade-off' (Heil *et al.*, 2001), and describes the requirement of a substantial amount of energy for the induction of SAR in response to chemical elicitors, resulting in reduced plant growth. BTH-treated wheat exhibits reduced growth and decreased seed production in response to chemical elicitors, and the reduction in growth is

TABLE 1. *Abbreviations used in the text*

| | |
|---|---|
| BABA | DL-3-Aminobutyric acid |
| BTH | Benzothiadizole |
| *CaPR4* | *Capsicum annuum pathogenesis-related 4* |
| *CaPIN2* | *Capsicum annuum protease inhibitor 2* |
| *CaTin1* | *Capsicum annuum TMV-induced Clone 1* |
| cfu | Colony-forming unit |
| CMV | Cucumber mosaic virus |
| dpt | Days post-treatment |
| hpi | Hours post-inoculation |
| ISR | Induced systemic resistance |
| JA | Jasmonic acid |
| LOX | Lipoxygenase |
| MABA | *meta*-Aminobenzoic acid |
| NPR1 | Nonexpressor of PR genes 1 |
| OABA | *ortho*-Aminobenzoic acid |
| PABA | *para*-Aminobenzoic acid |
| PGPR | Plant growth-promoting rhizobacteria |
| SA | Salicylic acid |
| SAR | Systemic acquired resistance |
| TMV | Tobacco mosaic virus |
| *Xav* | *Xanthomonas axonopodis* pv. *vesicatoria* |

more significant under nitrogen-shortage conditions (Heil *et al.*, 2000). The reduction in growth was found to be due to allocation fitness costs resulting from the competing metabolic demand of plant-related compound synthesis (Heil, 1999). Following the initial report of this phenomenon, many groups have observed similar effects. However, the detailed molecular and biochemical mechanisms underlying SAR are as yet unknown (Heil *et al.*, 2001). The possible molecular mechanisms underlying this effect were suggested to involve over-expression of phytotoxic compounds, in particular phenol-containing chemicals, which are toxic to the plant. To overcome the pitfall, several researchers in the academic and industrial fields have attempted to identify compounds that induce SAR without affecting plant growth in the field (Cipollini and Heil, 2010). However, there are no reports describing the identification of such chemicals for application under field conditions. Recent studies show that a form of defence priming occurs during the expression of SAR (Hammerschmidt, 2009). Defence priming is the enhancement of the basal level of resistance in plants, resulting in a faster and stronger resistance response following subsequent pathogen attack (Conrath *et al.*, 2006). Defence priming can be regarded as an efficient mechanism to manipulate the 'trade-off' machinery, resulting in minimizing the allocation fitness cost (Cipollini and Heil, 2010).

More recently, a new role for vitamins as chemical inducers of SAR has received attention owing to their safety and cost-effectiveness (Lyon, 2007). Three vitamins, vitamin $B_1$ (thiamine), vitamin B2 (riboflavin) and vitamin $K_3$ (menadione), induce SAR against fungal, bacterial and viral infections in arabidopsis, rice, cucumber, tobacco and tomato (Dong and Beer, 2000; Ahn *et al.*, 2005). Thiamine activates the expression of SAR-related genes in rice, tobacco and vegetable crops, improving the resistance of the plants to several pathogens (Ahn *et al.*, 2005). Riboflavin is produced by many species of plants and bacteria and is reported to effectively promote plant defence mechanisms against a biotrophic pathogen, tobacco mosaic virus (TMV), and the hemi-biotrophic pathogens, *Phytophtora parasitica* and *Ralstonia solanacearum* (Dong and Beer, 2000; Liu *et al.*, 2010; Taheri and Tarighi, 2011). A necrotrophic pathogen, *Botrytis cinerea*, was used as a model pathogen to examine the effect of riboflavin on the induction of SAR in bean and tomato. Exogenous application of 10–1000 μM riboflavin did not affect tomato plant resistance to *B. cinerea*, or hydrogen peroxide accumulation or lipoxygenase (LOX) activity, but successfully protected bean plants and enhanced LOX activity and hydrogen peroxide accumulation; this suggests plant-specific responses (Azami-Sardooei *et al.*, 2010). Interestingly, the pharmaceutical application of jasmonate elicited SAR against *B. cinerea* and enhanced the activity of LOX, which catalyses the first step of the production of jasmonase and its derivatives (Azami-Sardooei *et al.*, 2010). Collectively, these results suggest that riboflavin can potentiate defence reactions in bean plants by inducing hydrogen peroxide production and activating the LOX pathway, thereby eliciting SAR against necrotrophic pathogens such as *B. cinerea*. *Para*-aminobenzoic acid (PABA) is a cyclic amino acid that belongs to the vitamin B group and is also referred to as vitamin $B_x$. PABA is synthesized by bacteria, yeast and plants (Maki and Takeda, 1985). In medicine, it is mostly used as a protective drug against skin disorders and skin tumours caused by UV-exposure, such as Peyronie's disease, under the trade name 'Potaba' (Osgood *et al.*, 1982). PABA is necessary for the synthesis of folic acid, an irreplaceable vitamin B group component. PABA cannot be synthesized by mammals, but it is supplied by food and symbiotic bacteria that continuously produce PABA (Maki and Takeda, 1985). Certain previously unknown properties of PABA have recently been reported. PABA activates the synthesis of interferon, which has an important antiviral effect (Akberova, 2002). In China, PABA was reported to control fungal disease, but the induction of resistance was not studied in detail (Kelman and Cook, 1977). The potential role of PABA in elicitation of SAR against bacterial and viral pathogens has not been investigated to the best of our knowledge.

Based on our previous work on the induction of SAR by chemical compounds derived from bacterial metabolites, we searched for volatile compounds possessing greater SAR-inducing activity than *ortho*-aminobenzoic acid (OABA) for large scale field application (Yang *et al.*, 2011). Moreover, because OABA did not significantly elicit SAR in pepper plants, there was a demand to isolate a large number of bacterial volatiles and their derivatives. In the current study, OABA, *meta*-aminobenzoic acid (MABA) and PABA were screened for their capacity to induce SAR against *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) in pepper seedlings grown under greenhouse conditions. These compounds were used at two different concentrations (10 mM and 100 μM) and PABA showed significant reduction of symptom development in the field. To verify the induction of plant defence reactions by PABA, defence priming was examined by assessing the expression of the three marker genes, *Capsicum annuum pathogenesis-related 4* (*CaPR4*) gene for SA signalling (Yang *et al.*, 2009) *Capsicum annuum pathogenesis-related 9* (*CaPR9*) for SA (Lee *et al.*, 2012), *Capsicum annuum protease inhibitor 2* (*CaPIN2*) for jasmonic acid (JA) signalling (Wang *et al.*, 2012; Song *et al.*, 2005) and *Capsicum annuum TMV-induced Clone 1* (*CaTin1*) for ethylene signalling (Shin *et al.*, 2003) immediately after

pathogen challenge. Interestingly, a significant reduction in the incidence of diseases caused by cucumber mosaic virus (CMV) and *Xav* was observed at the end of the growing season. The yield of pepper plants treated with PABA was significantly increased compared with water control treatment. The present results demonstrate the effective protection of pepper plants from naturally occurring bacterial and viral pathogens by the folate precursor, vitamin $B_x$.

## MATERIALS AND METHODS

### *Plant preparation and greenhouse experiment*

Pepper plants (*Capsicum annuum* L. 'Bukwang') were cultivated in a growth chamber at 25 °C under a 16 h/8 h light/dark photocycle. Disease assay and plant preparation were carried out as previously described (Kang *et al.*, 2007). For pathogen challenge, a culture of the compatible bacterial pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) ($OD_{600}$ = 0.04 in 10 mM $MgCl_2$) was pressure-infiltrated into pepper leaves using a needleless syringe 1 week after drench-application of the chemicals PABA (Sigma-Aldrich, Milwaukee, WI, USA) to the pepper roots, as described previously (Oh *et al.*, 2006; Kang *et al.*, 2007). The severity of symptoms was scored from 0 to 5 as follows: 0 = no symptoms; 1 = yellowish colour; 2 = chlorosis only; 3 = partial necrosis and chlorosis; 4 = necrosis of the inoculated area and expanded chlorosis; 5 = complete necrosis of the inoculated area. Bacterial pathogens were cultured overnight at 28 °C in LB medium supplemented with the appropriate antibiotics. Chemical treatment of pepper roots was performed as described previously (Kang *et al.*, 2007). As a positive control, roots were treated with 0.5 mM BTH. Leaves were harvested at the indicated times and then frozen immediately in liquid nitrogen for total RNA extraction. Intact pepper leaves were used for non-stress treatments. Following inoculation with pathogen, plants were returned to the growth chamber and leaf tissue was harvested 0, 3 and 6 h after inoculation with *Xav*, and then used for isolation of total RNA.

### *Field trial*

The field trial was conducted at the Cheongwon-gun, Chungcheongbuk-do, Korea (36°35′ 32.27′N, 127°30′34.75′E) in spring 2011. Pepper seeds (*C. annuum* 'Bukwang') were surface-sterilized using 5 % sodium hypochlorite for 10 min, and rinsed five times with sterile distilled water. The seeds were then placed on Murashige and Skoog medium (MS; 0.22 % MS salt including vitamins, 1.5 % sucrose and 0.8 % plant agar, pH 5.8) in a transparent sterile container. The pepper seedlings were grown in a growth chamber at 25 °C under dark condition. Germinated pepper seeds were transferred to the sterilized soil that contained a low level of nutrient soil-less mixture (Punong Co. Ltd, Gyeongju, Korea) and cultivated for 3 weeks in greenhouse. For testing SAR ability under field conditions, pepper seedlings were soaked in 0.5 mm BTH solution for 1 h and transplanted at a distance of 40 cm apart in the field. The same volumes of sterilized water were used as a negative control. Before transplanting, each row was covered with black and white polyethylene plastic film. Treated peppers were grown on in 20-cm-high beds which were 30 × 880 cm wide. Single-row treatment plots were replicated four times in a completely randomized design and consisted of 23 plants.

### *Induced resistance against* Xav

For field testing for SAR, 10 d after application of PABA, bacterial suspensions of $10^6$ colony-forming units (cfu) $mL^{-1}$ *Xav* were used for penetration of the abaxial surface of pepper leaves using the needleless syringe method (Doo Won Meditec Co., Kim Je, Korea). Seven days after the pathogen challenge, disease severity was assessed as described previously (Yang *et al.*, 2009). The severity of symptoms on the leaf was scored from 0 to 5: 0 = no symptoms; 1 = mild chlorosis; 2 = chlorosis only; 3 = chlorosis and mild necrosis; 4 = necrosis; 5 = severe necrosis of the inoculated area. The experiment was repeated four times with ten replications (one plant per replication).

### *RT-PCR and quantitative RT-PCR (qRT-PCR)*

Real-time PCR was performed with a Bio-Rad CFX96. Total RNA was isolated from pepper leaf tissues using Tri reagent (Molecular Research Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out with 2 μg of DNase-treated total RNA, oligo-dT primers and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT; Enzynomics, Dajeon, Korea). PCR reactions were carried out according to the manufacturer's instructions. The expression of candidate priming gene was analysed using the following primers: 5′-AGCCTGAAATAGAAGAAACGGAGATGGAGATGAG A-3' (*CaTin1*-F), 5′-GGAACCAGAATTGGTTACTCATGGC TACCTGAAC-3′ (*CaTin1*-R), 5′-TGGGACTTTCATTTGTG AAGGAGAG-3′ (*CaPIN2*-F), 5′-GACACAGTGAATAGGC AATATTTGG-3′ (*CaPIN2*-R), 5′-AACTGGGATTGAGAAC TGCCAGC-3′ (*CaPR4*-F), 5′-ATCCAAGGTACATATAGAG CTTCC-3′ (*CaPR4*-R), 5′-GACTAGTTTCAAGAGCATCA-3′ (*CaPR9*-F), 5′-AATTGTATAGCCTGTAGCTG-3′ (*CaPR9*-R). As a control to ensure that equal amounts of RNA were analysed in each experiment, we also analysed *CaActin* using the primers 5′-CACTGAAGCACCCTTGAACCC-3′ and 5′-GAGACAACA CCGCCTGAATAGC-3′ (Wang *et al.*, 2012). Candidate priming genes were amplified from 100 ng of cDNA by PCR using an annealing temperature of 55 °C. A Chromo4 real-time PCR system (Bio-Rad Inc., Hercules, CA, USA) was used to carry out qRT-PCR. Reaction mixtures consisted of cDNA, iQ™ SYBR® Green Supermix (Bio-Rad Inc.) and 10 pM each primer. The thermocycle parameters were as follows: initial polymerase activation, 10 min at 95 °C; then 40 cycles of 30 s (s) at 95 °C, 60 s at 55 °C and 30 s at 72 °C. Conditions were determined by comparing threshold values in a series of dilutions of the RT product, followed by a non-RT template control and a non-template control for each primer pair. The relative RNA quantification, calculated using the 2-ΔΔCT method, together with standard errors of mean values among replicates were conducted using Bio-Rad manager (version 2.1) (Bio-Rad CFX Connect). Student's *t*-test was carried out to determine statistically significant differences between treated and untreated samples. If *P*-values $<0.05$, we considered the

target genes as differentially expressed. Relative RNA levels were calibrated and normalized against levels for the *CaA ctin* mRNA (GenBank accession no. AY572427).

### *Measurement of plant growth parameter*

To test whether BTH can inhibit plant growth under field conditions, after application with BTH solution, growth parameters such as weight were measured at the first harvest time. Additionally, to assess the effect of PABA on fruits yields, we harvested the fruits at 64 and 77 d post-treatment (dpt). The yield, as total fruit weight per treatment per plant, was measured and the experiment repeated four times.

### *Diagnosis of viral disease*

For viral diagnosis, test samples were selected from areas of the plant that exhibited symptoms of disease. Samples were ground and 50 mM $NaHPO_4$ (pH = 7.0) buffer was added. An ImmunoStrip (Agdia Inc., Elkhart, IN, USA) was inserted into the ground sample and buffer mixture for 30 min. To confirm CMV infection, we employed a RT-PCR technique with a specific primer for CMV CP, 5′-CGTTGCCGCTA TCTCTGCTAT-3′ and 5′-GGATGCTGCATACTGACAAA CC-3′. As a control to ensure that equal amounts of RNA were analysed in each experiment, *CaActin* was also analysed using the primers 5′-CACTGAAGCACCCTTGAACCC-3′ and 5′-GA GACAACACCGCCTGAATAGC-3′, which were designed based on the GenBank database sequence (GenBank ID: AY572427.1).

### *Direct inhibition assay*

To test whether PABA directly has an inhibitory effect against *Xav*, the bioassay was carried out using a paper disc assay method (De Beer and Sherwood, 1945). One hundred microlitres of $10^8$ cfu $mL^{-1}$ *Xav* suspension was spread onto King's B media. Fifty microlitres of 100 μM, 10 mM and 1 mM PABA were pipetted onto paper discs. Dried paper discs were transferred aseptically onto the surface of the growth medium. Two days later, we checked the development of the zones of inhibition. At least three replicate plates were prepared for the assay.

### *Statistical analysis*

Analysis of variance for experimental datasets was performed using JMP software version 5.0 (SAS Institute Inc., Cary, NC, USA; www.Sas.com). Significant effects of treatment were determined by the magnitude of the *F*-value ($P = 0.05$). When a significant *F*-test was obtained, separation of means was accomplished by Fisher's protected LSD at $P = 0.05$.

## RESULTS

### *Induction of systemic resistance by folic acid precursors*

As in our previous study (Yang *et al.*, 2009), the present study tested two concentrations of the folic acid precursors 2-aminobenzoic acid (OABA), 3-aminobenzoic acid (MABA) and 4-aminobenzoic acid (PABA) for their capacity to induce SAR against bacterial spot disease caused by *Xav* in pepper (Fig. 1A). Of the six treatments administered, only PABA elicited SAR (Fig. 1B). Drench application of 10 mM and 100 μM PABA reduced disease severity by 27- and 35-fold, respectively, compared with the controls (Fig. 1B). Treatment with 0.5 mM BTH prevented symptom development in pepper plants infected with *Xav* (Fig. 1B). Next, we used a lower population density ($10^4$ cfu $mL^{-1}$) to determine the specificity of three chemicals responsible for eliciting plant defence responses. Of four PABA concentrations tested (1 mM, 10 μM, 100 nM and 1 nM) only treatment with 1 mM

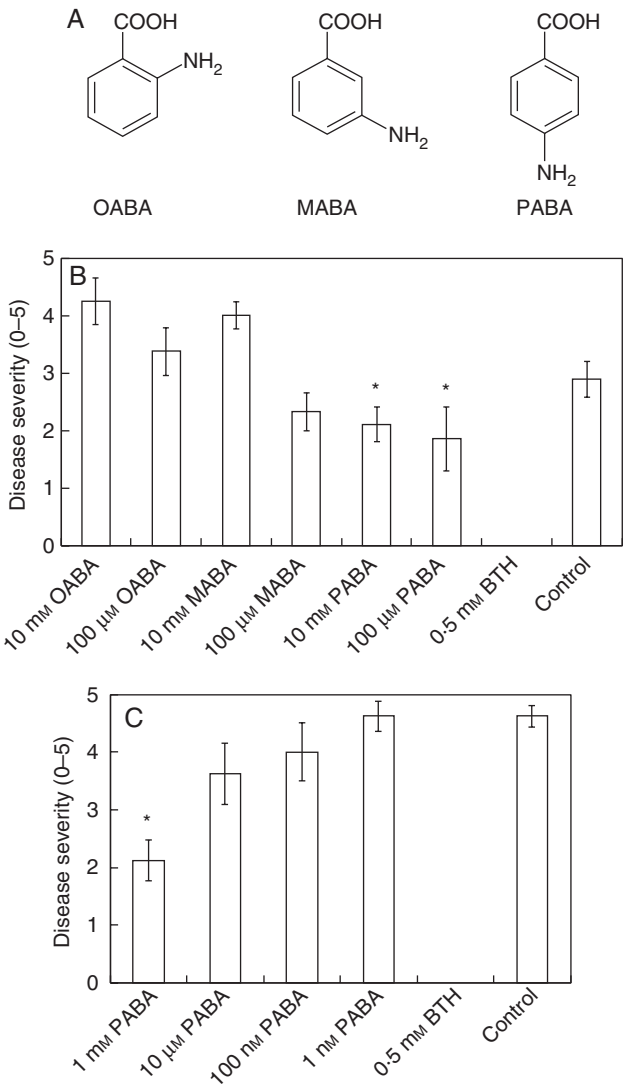


FIG. 1. Screening of folate precursors for the induction of systemic resistance in pepper plants under greenhouse conditions. (A) The chemical structures of OABA, MABA and PABA are shown. (B) The folate precursors were inoculated into the potting media containing 3-week-old pepper seedlings at concentrations of 10 mM and 100 μM. (B and C) Disease severity (0–5) of pepper seedlings treated with folate precursors at 10 mM and 100 μM PABA was assessed 7 d after infection with *X. axonopodis* pv. *vesicatoria* at $10^4$ and $10^6$ cfu $mL^{-1}$. Water and 0.5 mM BTH were used as negative and positive controls, respectively. Asterisks in (B) and (C) indicate statistically significant differences compared with water-treated control plants ($P = 0.05$). Error bars indicate the s.e.

PABA elicited a significant defence response (Fig. 1C). Assessment of SAR induction under high disease pressure conditions (infiltration of *Xav* at $10^6$ cfu $mL^{-1}$) showed a disease severity of 4.63 in control plants and 2.88 in BTH-treated plants. The PABA concentrations were selected to produce an effective response when applied in the field, and 1 mM PABA was found to consistently induce SAR against *Xav*. Notably, the effect of different compounds on the induction of SAR was plant species-dependent. Our previous results indicated that OABA elicited SAR in tobacco but not in pepper, despite the fact that both species belong to the Solanaceae family (Yang *et al.*, 2011). More interestingly, MABA did not show any effect on the defence reactions and growth in pepper plants, but was severely toxic to tobacco plants (Fig. 1B, and data not shown). Only PABA elicited SAR in both tobacco and pepper.

### *SAR and defence priming induction by PABA in the field*

To evaluate whether PABA induced SAR under field conditions, we examined plants for symptoms of bacterial spot disease 5–10 d after leaf-infiltration. At 20 and 30 dpt, the disease severity in plants treated with 1 mM PABA and water (control) was 1.69 and 1.50, respectively, whereas it was 2.57 and 2.66, respectively, in control-treated plants (Fig. 2A). The disease severity values at 20 and 30 dpt after drench application of BTH were 0.04 and 0.11, respectively (Fig. 2A). However, at 40 dpt, there were no differences between PABA and control-treated plants, whereas BTH consistently reduced the symptoms of bacterial spot disease (Fig. 2A). To confirm the induction of SAR and defence priming by PABA, the expression of the defence-related genes *CaPR4* and *CaPR9* for SA signalling, *CaPIN2* for jasmonic acid signalling, and *CaTin1* for ethylene signalling after 0 and 6 h of pathogen challenge was examined by qRT-PCR at 20 and 30 dpt. At 20 dpt, *CaPR4* gene transcription showed a 16-fold increase from 0 h post-inoculation (hpi) to 6 hpi with PABA, while a 7.1-fold increase was detected in control plants (Fig. 3; *CaPR4* panel). At 30 dpt, the transcription level of *CaPR4* at 6 hpi was up-regulated 2.2-fold by PABA treatment compared with that at 0 h, whereas the control was up-regulated 1.2-fold (Fig. 3; *CaPR4* panel). At 30 dpt, the transcription level of *CaPR9* was up-regulated 9.6-fold by PABA treatment, whereas the control was up-regulated 7.9-fold (Fig. 3; *CaPR9* panel). On 6 hpi at 30 dpt, both SA marker genes, and the mRNA levels of *CaPR4* and *CaPR9* significantly increased compared with water control. The relative expression levels of the *CaPIN2* and *CaTin1* genes in PABA treatment were reduced 2.6-fold at 20 dpt and 2-fold at 30 dpt, respectively, relative to that of the control treatment 6 h after pathogen challenge (Fig. 3; *CaPIN2* and *CaTin1* panels). No difference was detected between treatments for *CaPIN2* expression at 30 dpt and *CaTin1* at 20 dpt. The target virus was identified as CMV by enzymatic and virus-specific primer (CMV 2b)-based PCR (Fig. 2B).

### *PABA-elicited SAR against naturally occurring bacterial spot disease and CMV infection*

Severe disease symptoms were observed at the end of the pepper growing season, in mid-September, which became

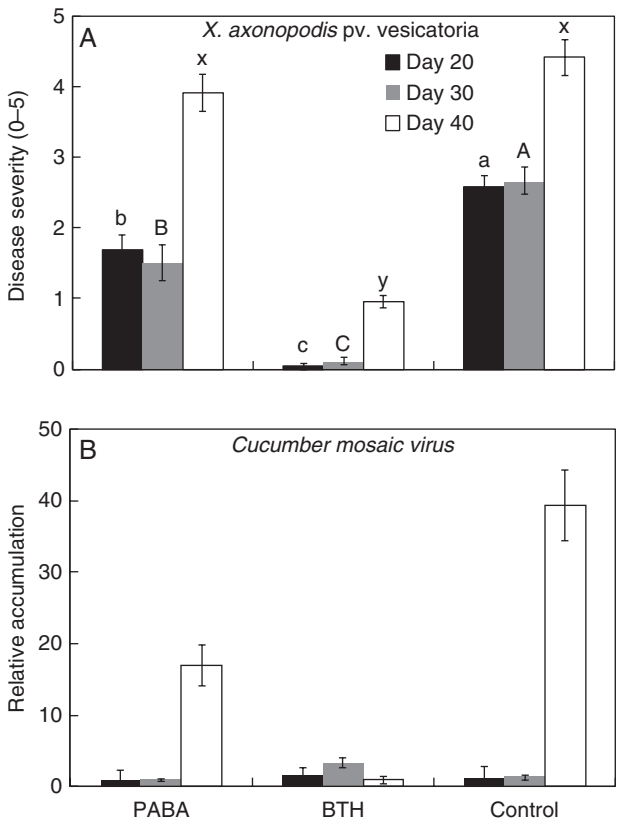


FIG. 2. Induction of systemic resistance by 1 mM PABA under field conditions. (A) Disease severity was measured at 20, 30 and 40 d after *X. axonopodis* pv. *vesicatoria* challenge at $10^6$ cfu $mL^{-1}$ in plants pretreated with 1 mM PABA and 0.5 mM BTH. Bars represent the mean ± s.e. (sample size, $n = 12$ replications per treatment). Different letters indicate significant differences between treatments in each time point ($P = 0.05$ according to least significant difference). The experiment was repeated four times with similar results. (B) The expression of coat protein genes was measured 20, 30 and 40 d after treatment with PABA, BTH and the control in pepper plants. Bars represent the mean value ± s.e. ($n = 3$). The housekeeping gene *CaActin* was used as a control. The experiment was repeated twice with similar results.

worse as a consequence of the unusually high temperature and long rainy season in Korea during 2011. Examination of the plants revealed spots, speck, mosaic and shoe-string symptoms, which are characteristic of bacterial spot disease caused by *Xav* and infection by TMV or CMV. Biochemical assays and PCR analysis identified the causative bacteria as *Xav*; this was based on 16s rRNA data, colony colour and morphology on semi-selective agar media and pathogenesis test in pepper plants and *Nicotiana benthamiana* (hypersensitive response) (data not shown). At 77 dpt, the bacterial spot symptoms were measured according to disease severity (using SAR assays as before). Bacterial numbers were quantified on leaf discs incubated in semi-selective media for *Xanthomonas* spp. The disease severity was 1.1 in PABA-treated plants, 1.3 in BTH-treated plants and 2.3 in the untreated controls (Fig. 4A). PABA and BTH application significantly reduced symptom development compared with that in the water control (Fig. 4A). The bacterial numbers were $\log_{10}$

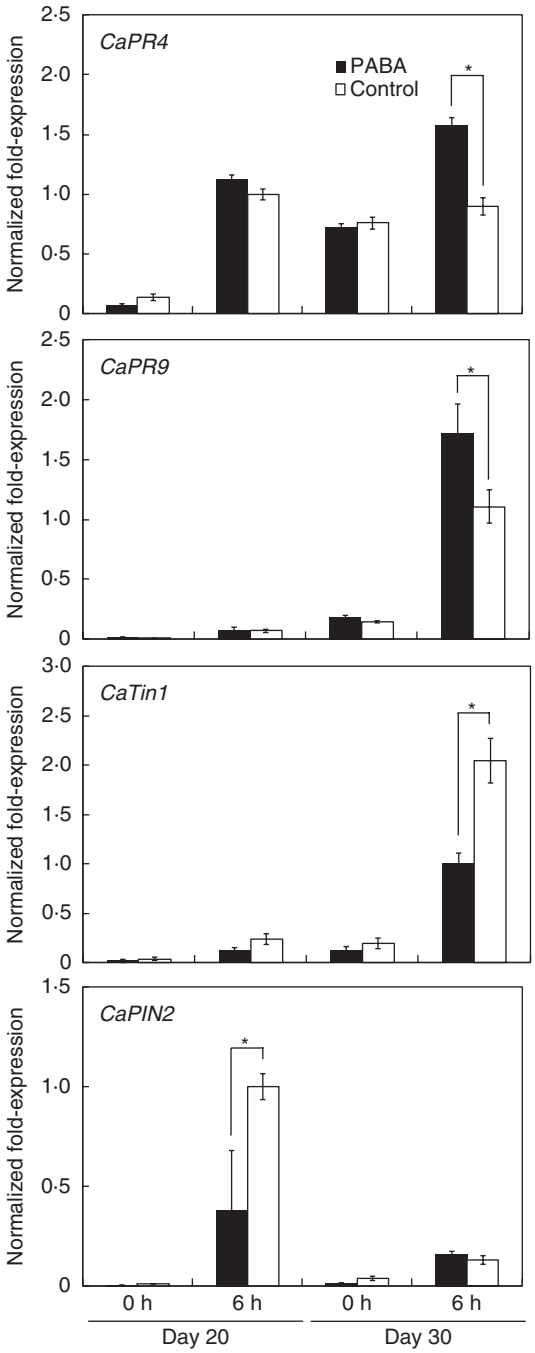


FIG. 3. Transcriptional expression of defence-related genes. The expression levels of the pepper resistance genes *CaPR4*, *CaPR9*, *CaTin1* and *CaPIN2* were assessed by qRT-PCR. Bars represent the mean value ± s.e. ($n = 3$). The housekeeping gene *CaActin* was used as a control. The experiment was repeated twice with similar results.

7.6, 7.2 and 8.4 cfu/leaf disc for PABA, BTH and control treatments, respectively (Fig. 4B). The bacterial population in PABA-treated plants was significantly ($P = 0.05$) lower than in control plants, but higher than that in BTH-treated plants (Fig. 4B). The target virus was identified as CMV by enzymatic and virus-specific primer-based PCR (Fig. 4D). The CMV 2b RNA accumulation in control plants at 77 dpt was 55 % higher than that in PABA-treated pepper plants (Fig. 4D). CMV accumulation was significantly different between BTH and water-treated plants (Fig. 4D). The disease symptoms were evaluated based on a disease severity score from 0 to 5. At 77 dpt, plants pre-treated with PABA showed a disease severity score of 0.8, while BTH and control plants showed disease severity scores of 2.3 and 2.4, respectively (Fig. 4C).

### *PABA increases plant yield*

Previous studies suggested that the induction of SAR results in the inhibition of plant growth by a mechanism referred to as 'allocation fitness cost'. In the current study, shoot length was measured at 64 dpt (data not shown) and fruit yield was assessed at 64 and 77 dpt. There were no differences in shoot length between PABA- and control-treated plants (data not shown). Consistent with previous studies, BTH significantly reduced shoot length (data not shown). Fruit yield was similar in PABA-treated and control plants at the first harvest (64 dpt), but was higher in PABA-treated plants at the second harvest (77 dpt) (Fig. 5). The accumulative yield of PABA-treated plants was higher than that of BTH-treated plants and the untreated controls (Fig. 5). The total weight of pepper fruit per plant was 264.9 g for PABA, 20.2 g for BTH and 217.5 g for the controls (Fig. 5). These results indicate that PABA did not affect plant vegetative growth as reflected by shoot length, but increased the yield of the pepper plants.

## DISCUSSION

Following the hypersensitive response elicited by necrotizing pathogens, plants can also activate SAR (Hammerschmidt, 2009). Various chemicals are also reported to trigger plant defence responses (Lyon, 2007). Among those eliciting SAR in plants, vitamins have received attention as SAR inducers because they are cost effective and safe for the environment. In the present study, we identified a new component of the vitamin B group capable of inducing SAR against bacterial and viral diseases in pepper plants under field conditions. By contrast to the chemical control of plant pathogens, a SAR-based plant protection strategy is advantageous because of its long-lasting and broad-spectrum effects on managing plant pathogens. Currently used agro-chemicals have a specific target organism and their effect is maintained from a few days to a few weeks. BTH, for example, protects plants against more than ten pathogens for up to 10 weeks. Despite rapid progress in the development of agro-chemicals, most of the compounds identified to date are fungicides. The management of bacterial pathogens requires the use of antibiotics, which are associated with the appearance of antibiotic-resistant bacterial strains by spontaneous mutation or acquisition of resistant genes from other species. Furthermore, climate change is associated with the recent appearance of several viral diseases that have led to serious problems worldwide. The transformation of the viral genome, breeding of resistant lines, and the control of insect vectors are currently the only known methods for controlling viral diseases.

In this study, we showed that PABA can induce SAR against plant pathogenic bacteria and viruses. PABA is classified as a

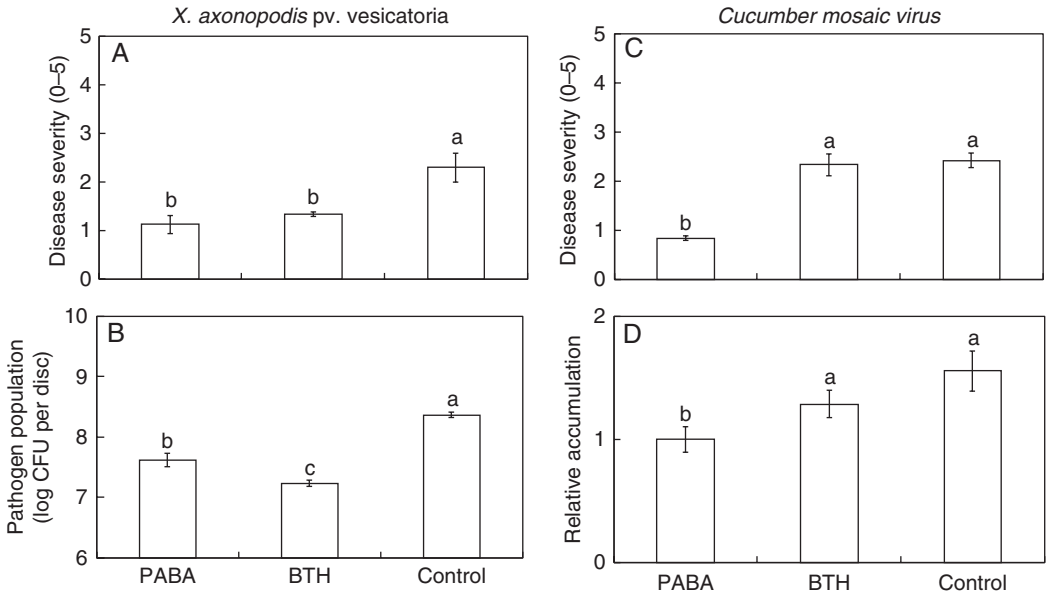


FIG. 4. PABA elicited SAR against naturally occurring bacterial pathogens. (A, B) Disease severity (0–5) and a population of the naturally occurring pathogen *X. axonopodis* pv. *vesicatoria* at 77 dpt. (C, D) Disease severity (0–5) and expression of the CMV coat protein gene at 77 dpt. Water and 1 mM BTH were used as negative and positive controls, respectively. Four independent experiments were performed with ten pepper plants per treatment. Different letters indicate statistically significant differences compared with water-treated control plants ($P = 0.05$). Error bars indicate the s.e.

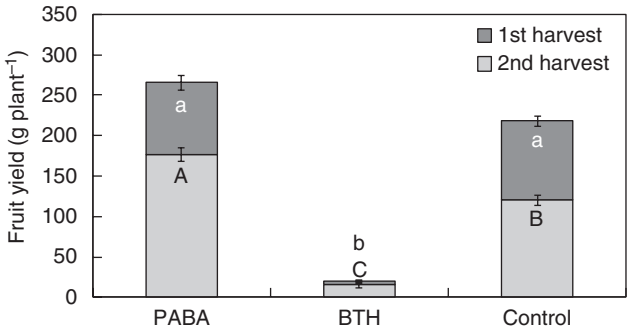


FIG. 5. Increase in pepper yield induced by PABA. Fruit yield of PABA- or control-treated pepper plants was assessed at 64 and 77 dpt. Water and 1 mM BTH were used as the negative and positive controls, respectively. Different letters indicate statistically significant differences compared with water-treated control plants ($P = 0.05$). Error bars indicate the s.e.

vitamin $B_x$ (Maki and Takeda, 1985). Certain bacteria, including *Lactococcus lactis* and *Bacillus subtilis*, are able to synthesize folic acid from PABA, which is therefore considered a vitamin in these organisms (Sybesma *et al.*, 2003; Zhu *et al.*, 2005). However, mammals cannot synthesize folic acid from aminobenzoic acid; thus, the use of PABA is safe. Previous studies examined the effects of three vitamins from the vitamin B complex group, vitamin $B_1$ (thiamine), vitamin $K_3$ (menadione) and $B_2$ (riboflavin) (Lyon, 2007), which were shown to elicit systemic resistance in different plant species (Dong and Beer, 2000; Sybesma *et al.*, 2003; Ahn *et al.*, 2005, 2007; Zhu *et al.*, 2005; Azami-Sardooei *et al.*, 2010; Liu *et al.*, 2010; Taheri and Tarighi, 2010). The induction of SAR by thiamine was reported to be mediated by SA- and $Ca^{2+}$-related pathways (Ahn *et al.*, 2005). The same authors also showed that thiamine induces SAR by priming plants through the regulation of NPR1 (nonexpressor of PR genes 1) expression and accumulation of hydrogen peroxide (Ahn *et al.*, 2007). Similarly, riboflavin-elicited SAR is mediated by protein kinase and NPR1-dependent signalling pathways without SA accumulation in tobacco and arabidopsis (Dong and Beer, 2000). In rice, the application of riboflavin increases systemic defence responses against *Rhizoctonia solani* (Taheri and Tarighi, 2010). qRT-PCR studies show that the priming effect of riboflavin is mediated via up-regulation of LOX transcription. The JA-deficient rice mutant, *hebiba* and LOX inhibitors compromise the effect of induced resistance. In the present study, defence priming by PABA treatment was demonstrated by the up-regulation of the *CaPR4* and *CaPR9* genes in pepper, which are markers of SA signalling (Yang *et al.*, 2009) (Fig. 3; Supplementary Data Fig. S1). After 6 hpi, the expression of *CaPIN2* at 20 dpt and *CaTIN1* at 30 dpt was down-regulated by PABA treatment, indicating that cross-talking between defence signalling was occurring. Previously SA and JA signalling pathways were reported to be antagonized (Kunkel and Brooks, 2002). In sugar beet, riboflavin increases plant basal defence responses, including hydrogen peroxide accumulation and the up-regulation of the cationic peroxide and phenylalanine ammonia-lyase genes, which leads to the production of phenolic compounds and cell-wall lignification in response to *R. solani* infection through the root (Taheri and Tarighi, 2011). In tobacco, riboflavin application protects seedlings against *Ralstonia solanacearum* and *Phytophthora parasitica* by inducing an oxidative burst response, the expression of defence-related genes, and the accumulation of phenolic compounds such as scopoletin and lignin (Liu *et al.*, 2010). To the best of our knowledge, the elicitation of SAR against viral

disease by a vitamin has not been reported previously. Furthermore, our experiments were conducted in the field, and examined SAR against naturally occurring diseases. BTH-induced SAR significantly reduced the symptoms of CMV infection during the early cultivation period in our study, but was ineffective at the end of the season (Figs 2 and 4). Importantly, PABA-elicited SAR enhanced fruit yield in pepper as well as protection of the plant against bacterial and viral pathogen infection (Fig. 5). A number of studies have reported that chemical SAR inducers like BTH, SA and BABA have a negative effect on plant growth and yield (Heil *et al.*, 2000; Dietrich *et al.*, 2004; Aleandri *et al.*, 2010). In the current study, we observed an almost complete inhibition of fruit production under field conditions, in contrast to PABA application (Fig. 5).

CMV is one of the most destructive agents affecting crop yields worldwide, and the induction of resistance against CMV infection has been attempted by biological and chemical means (Murphy, 2006). Although a variety of methods are currently used for controlling CMV infection, they have not been successful under field conditions. Two resistance genes have been identified in arabidopsis and common bean (*Phaseolus vulgaris* 'Othello'), suggesting that promoting resistance by classical breeding is difficult (Takahashi *et al.*, 2002; Seo *et al.*, 2006).

In the present study, PABA induced resistance against CMV (Figs 2B and 4C, D). The elicitation of induced resistance to CMV has been attempted using several biological agents, including plant pathogens and PGPR. Inoculation of the lower first and second leaves with pathogenic *Collectotrichum orbiculare* and *Pseudomonas syringae* pv. *lachrymans* reduced the number of lesions and delayed systemic symptom development on a third leaf subsequently challenged with CMV (Bergstrom *et al.*, 1982). In addition to pathogenic fungus- and bacterium-induced resistance against CMV, cucumber seedlings inoculated with PGPR strains 90–166 and 89B61 showed a delay in the development of CMV symptoms of up to 7 d relative to control plants (Raupach *et al.*, 1996). In another study, four PGPR strains showed a positive effect on ISR against CMV in tomato under greenhouse conditions (Zehnder *et al.*, 2000), but were not effective under high disease pressure by CMV in the field. In field trials with PGPR strains in cucumber, successful protection of plants inoculated with CMV was achieved. Tomato plants treated with PGPR plus chitosan showed a similar response to CMV infection to that of age-related resistance (Murphy *et al.*, 2003). The same PGPR preparation elicited ISR against CMV in arabidopsis (Ryu *et al.*, 2007). The mechanism underlying PGPR-mediated ISR was shown to involve the JA signalling pathway, but was independent of SA and NPR1 signalling, as demonstrated in arabidopsis wild-type and mutant lines (Ryu *et al.*, 2004). Plant and bacterial extracts elicit SAR against CMV as below. Crip-31, a protein extracted from *Clerodendrum inerme*, reduced systemic accumulation of CMV in tobacco (Praveen *et al.*, 2001). The spray inoculation of exopolysaccharides produced by *Serratia* sp. strain Gsm01 protected tobacco plants against CMV infection (Ipper *et al.*, 2008). As described previously, PABA can be synthesized in several microorganisms, such as *Bacillus subtilis*, *Lactobacillus lactis* and *L. gasseri* (Wegkamp *et al.*, 2004, 2007; Zhu *et al.*, 2005).

Our results indicate that PABA did not cause complete inhibition of CMV accumulation in the leaves. Interestingly, CMV accumulation did not differ significantly between PABA-treated and control plants during the early season or at 20 and 30 dpt as detected by qRT-PCR (Fig. 2B). After 40 dpt, CMV RNA levels were 2-fold lower in pepper plants treated with PABA than in control plants (Fig. 2B) and this effect was maintained until harvest time at 105 dpt (Fig. 4D). Only four chemicals have induced plant defence responses against CMV infection in previous studies. In cantaloupe, pre-treatment with acibenzolar-*S*-methyl (BTH) reduced systemic movement of CMV (Smith-Becker *et al.*, 2003). In tobacco and arabidopsis, SA triggered induced resistance against CMV by inhibiting systemic movement and replication (Naylor *et al.*, 1998; Mayers *et al.*, 2005). In the same system, the induction of resistance against CMV did not involve the inhibition of systemic movement, indicating that the mechanisms underlying plant defence responses against CMV are species-specific (Mayers *et al.*, 2005). The restriction of systemic movement was observed in arabidopsis plants pre-treated with SA, but was absent in similarly-treated squash plants (Mayers *et al.*, 2005). Furthermore, SA and antimycin A, an inducer of the mitochondrial enzyme alternative oxidase, failed to induce systemic resistance against CMV in squash, but reduced the symptoms of CMV infection (Mayers *et al.*, 2005). Despite the effectiveness of several chemical triggers in protecting plants against CMV infection, their use under field conditions has not been reported. In tomato, BTH treatment reduced disease incidence and severity and delayed primary symptom development in the field. ELISA analysis indicated that BTH completely inhibited the systemic movement of the virus to newly developed leaves (Anfoka, 2000). To the best of our knowledge, the present study is the first to report the use of chemical triggers for the management of CMV in pepper in the field.

Different responses to folic acid derivatives have been reported in different plant species (Hoang *et al.*, 2007; Yang *et al.*, 2011). In arabidopsis, MABA and PABA did not affect root growth, whereas OABA significantly inhibited root growth in a dose-dependent manner (Hoang *et al.*, 2007). Under similar *in vitro* conditions, 1 mM MABA was sufficient to inhibit the growth of tobacco seedlings while 18 mM OABA and PABA were ineffective (Yang *et al.*, 2011). In the present study, treatment of pepper seedlings with three different folic acid derivatives did not result in growth inhibition in initial experiments (data not shown). More interestingly, drench application of OABA and PABA in tobacco reduced the symptoms of soft-rot disease caused by *Pectobacterium carotovorum* (also called *Erwinia carotovora*), and OABA had a stronger effect than PABA when used at low concentrations (1 mM) (Yang *et al.*, 2011). However, in the present study, only PABA protected pepper seedlings against *Xav* infection (Fig. 1B, C). Drop-inoculation with different concentrations of PABA did not inhibit the growth of *Xav* (data not shown). This result indicates that the reduction in the symptoms of *Xav* infection was the result of induced resistance rather than direct inhibition of bacterial growth, despite the possible translocation of introduced PABA from the root to the shoots and leaves.

In conclusion, the present study is the first to demonstrate the effective elicitation of SAR against bacterial and viral pathogens by a folate precursor under greenhouse and field conditions. Systemic defence signalling elicited by PABA mainly involved SA pathways.

## SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Figure S1: transcriptional expression of *Capsicum annuum defense-related 4* and *9* genes by SA and methyl jasmonate treatments.

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