Eur J Plant Pathol (2009) 125:119–130
DOI 10.1007/s10658-009-9464-9

Accumulation of H_2O_2 in xylem fluids of cucumber stems during ASM-induced systemic acquired resistance (SAR) involves increased LOX activity and transient accumulation of shikimic acid

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Received: 18 June 2008 / Accepted: 16 March 2009 / Published online: 14 April 2009

Abstract Systemic acquired resistance (SAR) to *Colletotrichum orbiculare* was induced in young cucumber (*Cucumis sativus*) plants within 3 h of ASM (acibenzolar-S-methyl) application onto the first leaves. A potent signal associated with significant accumulation of hydrogen peroxide in xylem fluids from severed stems appeared to be rapidly translocated from elicited lower leaves within 3 h and 6 h after treatment. Some metabolites of the shikimate, phenylpropanoid and lignin biosynthetic pathways were quantified and significant increases in the levels of shikimic acid were observed in ASM-treated plants challenge-inoculated with the anthracnose fungus. Furthermore, the expression of the 5-enolpyruvylshikimate-3-phosphate synthase gene (*EPSPS*) was 1.5 times higher within 12 h after ASM treatment in challenge-inoculated plants than in the untreated control. The involvement of lipoxygenase activity, shikimic acid and others such as caffeic acid in the induction of SAR is discussed.

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Keywords Anthracnose fungus · EPSPS · NADPH oxidation · Phenolics · ROS

Introduction

Systemic acquired resistance (SAR) can be induced by synthetic chemicals such as acibenzolar-S-methyl [ASM, benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester] (Ishii et al. 1999). The phenomenon of SAR suggests that a signal originates from the site of treatment with defence-inducing compounds or infection by pathogens and then translocates throughout the plant. Narusaka et al. (1999) carried out experiments using cucumber plants in which ASM-treated first leaves were detached at various times then whole plants were spray-inoculated with the scab fungus (*Cladosporium cucumerinum*) 24 h after ASM treatment. The rapid expression of peroxidase and chitinase genes was found in untreated upper leaves several hours after treatment with ASM. This indicated that some signal molecules were rapidly translocated from ASM-treated leaves to untreated upper leaves; however, the nature of these signals is unknown.

Early events occurring prior to the establishment of SAR include a change of plasma membrane potential, degradation of membrane lipid and an increase in membrane permeability. These events are likely to be involved in lipid metabolism, associated with lipoxygenase (LOX) activity (Siedow 1991). The products of

LOX enzyme may contribute to defence responses in plants, including that of direct inhibition of the pathogen and accumulation of phytoalexins (Alami et al. 1999).

Phenylpropanoid metabolism in plants leads to the formation of numerous phenolic compounds that have important functions in plant defence responses associated with wounding or pathogen infection. The level of phenylalanine ammonia lyase (PAL) activity is the key factor in the regulation of the phenylpropanoid pathway. Pre-treatment of cucumber plants with ASM increased resistance to cucumber anthracnose fungus and systemically primed the *PAL* gene expression for pathogen attack (Cools and Ishii 2002). By contrast, the inhibition of PAL activity prevented a typical resistance response in the host tissue to that of fungal infection. *PAL*-suppressed plants were more susceptible to fungal infection (Maher et al. 1994). Many secondary metabolites, including phenolics, isoflavonoid phytoalexins and lignins are derived from branches of the phenylpropanoid pathway. Thus, salicylic acid (SA) is instrumental in the plant's ability to mount a successful defence in response to pathogen attack.

The shikimate pathway may also play a crucial role in the regulation of phenylpropanoid metabolism. Many secondary aromatic metabolites such as lignin require shikimic acid as a precursor and during wound repair; increased levels of phenolic compounds derived from shikimate have been reported. For example, Fuchs and Vries (1969) have demonstrated that shikimic acid-U-^{14}C was directly incorporated into aromatic compounds with >50% of shikimic acid-U-^{14}C directly incorporated into the lignin found in a *Fusarium*-infected resistant variety of tomato. Incorporation of labelled shikimate decreased in the susceptible variety. Glyphosate is an inhibitor of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) that catalyses a key step in the biosynthesis of aromatic amino acids, which in turn are the substrates for the synthesis of phytoalexins and lignins but are also involved in the synthesis of SA. Keen et al. (1982) reported increased susceptibility to *Phytophthora megasperma* f. sp. *glycinea* and reduced glyceollin accumulation in soybean seedlings treated with the herbicide glyphosate.

ASM treatment of the first leaves and inoculation of whole cucumber plants with the anthracnose fungus caused the rapid accumulation of H_2O_2 below the penetration site on ASM-untreated third leaves (Park et al. 2002; Ishii et al. 2004). This might have contributed to the increased formation of lignin-like compounds at the same site and triggered defence responses against infection by *Colletotrichum orbiculare*. Hydrogen peroxide itself may also serve as a signal molecule or is perhaps a by-product of a signal molecule. In this study, we attempted to identify the signal molecule involved in ASM-induced systemic resistance in cucumber plants by monitoring the production of H_2O_2 in stems above ASM-treated leaves. Additionally, some key compounds of the shikimate, phenylpropanoid, and lignin biosynthetic pathways were quantified. The relative levels of *EPSPS* expression and LOX activity were also examined.

Materials and methods

Plant material and pathogens

Cucumber (*Cucumis sativus* cv. Shin Suyo Tsukemidori) was grown in a greenhouse at 25°C under natural light conditions. Cucumber plants at the three-leaf growth stage were used throughout the experiments in this study. The anthracnose fungus, *C. orbiculare* (isolate C-14, a gift from ZEN-NOH R&D Centre, Japan) was maintained on potato dextrose agar (PDA) and cultured on PDY (PDA amended with 0.5% yeast extract) to obtain abundant conidia for inoculation tests.

ASM treatment and anthracnose fungus inoculation

ASM treatment was performed as described by Ishii et al. (1999). The first true leaves of cucumber plants were dipped in aqueous ASM suspensions at a concentration of 100 μg (a.i.) ml^{-1} (water-dispersible granular formulation containing 50% active ingredient, kindly supplied by Syngenta AG) or distilled water (DW) for 5 sec. Each treatment consisted of three replicates. Three hours after treatment, whole plants were spray-inoculated with *C. orbiculare* conidial suspensions (ca. 5×10^5 conidia ml^{-1}) in DW and incubated in a moist chamber in the dark at 20°C for 24 h before being transferred to a greenhouse and kept at 25°C until disease assessment 7 days post-inoculation. Abbreviations for DW-treated and uninoculated, DW-treated and inoculated,

ASM-treated and uninoculated, and ASM-treated and inoculated experimental categories were DW U, DW I, ASM U and ASM I, respectively. The third leaf was collected from each cucumber plant at different time intervals (0 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h) after treatment and stored at −80°C until use. Three trials of each experiment were conducted.

Detection of hydrogen peroxide in xylem fluids

Hydrogen peroxide in the cucumber xylem fluids was measured by chemiluminescence in a ferricyanide-catalysed oxidation of luminol (Pedreira et al. 2004). At indicated time points (0 h, 3 h, 6 h, and 12 h after treatment) cucumber plants were moved to a humid box for 30 min to reduce the transpiration rate of the plants. Following this the stems were cut with a razor blade, between the second and third true leaves and the upper part of the plants removed. The first three drops of exudates from the cut stems were then blotted onto filter paper. The exudates collected from the cut surface of the lower part of the stems were used directly for the determination of H_2O_2. An aliquot (10 µl) of xylem fluids and 390 µl of a 50 mM potassium phosphate buffer solution (pH7.9) were mixed in a tube and placed in a luminometer (AB-2200 Luminescencer-PSN, ATTO, Japan). Initially, 50 µl of 0.25 mM luminol in 50 mM potassium phosphate buffer (pH7.9) was injected into the test tube, and after 5 s the reaction was started by injecting 50 µl of freshly prepared 14 mM $K_3Fe(CN)_6$. The chemiluminescence was integrated for 10 s after starting the reaction. Four trials of the experiments were conducted.

Lipoxygenase assay

Five frozen leaf discs (10 mm diam) were homogenised in a chilled pestle and mortar using 1 ml of 50 mM sodium phosphate buffer (pH6.5) containing 0.25% (v/v) Triton X-100, 1 % (w/v) polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF), based on the method used by Baracat-Pereira et al. (2001). The homogenate was centrifuged at 19,600 *g* for 30 min at 4°C, and the supernatant containing the enzyme extract collected and stored on ice. Concentrations of the proteins were then determined using the method described by Bradford (1976).

LOX activity was measured using a UV/Visible spectrophotometer (DU 800, Beckman, USA). Substrate solution containing linoleic acid (Wako, Japan) was prepared according to the protocol of Anthon and Barrett (2003). The optimum pH of LOX was determined using a range of pH values of the 50 mM buffers containing sodium acetate (pH4.6), sodium phosphate (pH5.0–8.0), HEPES (pH8.5) and sodium borate (pH9.4), respectively, with 0.4 mM linoleic acid as a substrate. Enzyme assays were prepared by the addition of 20 µl of enzyme extract to a mixture containing 20 µl of 20 mM linoleic acid (0.4 mM substrate in the final volume) in 960 µl of 50 mM HEPES buffer (pH8.5). All reactions were conducted at 25°C. LOX activities were determined by the increase in absorbance at 234 nm during a 2-min period. The same procedure was used with all the blank reactions containing HEPES buffer (pH8.5) which replaced the enzyme extract. The molar extinction coefficient of 25,000 M^{-1} cm^{-1} at 234 nm was used to calculate LOX activity and expressed as nkatals (the amount of enzyme that catalyses the conversion of one nanomole of substrate per second). The experiment was repeated three times.

Quantification of some key compounds in shikimate, phenylpropanoid, and the lignin biosynthetic pathways

The method described by Mattila and Kumpulainen (2002) was used to quantify phenolic compounds. Briefly, a freeze-dried cucumber leaf powder (0.1 g) was weighed into a 50-ml tube (SARSTEDT, Germany) and homogenised in 7 ml of a mixture of methanol [containing 2 g l^{-1} of 2,(3)-*tert*-butyl-4-hydroxyanisole (BHA)] and 10% acetic acid (85:15) using a Physcotron homogeniser (Nichion Irika, Japan). The homogenates were ultrasonicated for 30 min at 4°C, made up to 10 ml with DW, after which, 1 ml of the sample was taken for analysis to screen for free phenolic acids. Thereafter, 12 ml of DW and 5 ml of 10 N NaOH were added to the tubes; oxidation was prevented by replacing air with nitrogen and all samples were protected from light. After 16 h all the samples extracted were adjusted to pH 2, and partitioned using a mixture of cold diethyl ether (DE) and ethyl acetate (EA) in a 1:1 proportion to obtain liberated phenolic acids. The organic (DE-EA) layer was collected, evaporated and the residues were dissolved into 1 ml of methanol.

To quantify phenolic compounds, the Agilent 1100 Series HPLC system (Agilent Technologies, USA) was used and HPLC conditions were the same as described previously (Mattila and Kumpulainen 2002), except that a Wakopak® C_{18} reverse-phase column (4.6 mm×250 mm, Wako, Japan) was used. Ten microliters of the standards and sample extracts were applied to the HPLC system (35°C) with a gradient elution with 50 mM H_3PO_4, pH2.5 and acetonitrile as follows: (time [min]/acetonitrile [%]/flow [ml min^{-1}]=0/5/0.7, 5/5/0.7, 55/50/0.7, 65/50/0.7, 67/5/0.7, post-time 6 min). The concentrations of phenolic compounds were calculated from three trials of time-course experiments using a calibration curve of known quantities of the phenolic acid standards.

To quantify shikimic acid, the method described by Singh and Shaner (1998) was used. Plant samples were ground in liquid nitrogen with a pestle in a mortar, then further ground in a 1:10 (tissue weight/volume of 0.25 N HCl) ratio in the same mortar. The extract was centrifuged at 19,600 *g* for 15 min. An aliquot of the supernatant (50 µl) was mixed with 0.5 ml of 1% periodic acid to oxidise shikimic acid. Three hours later the sample was mixed with 0.5 ml of 1 N NaOH, and 0.3 ml of 0.1 M glycine. The solution was then thoroughly mixed, and the absorbance immediately measured at 380 nm.

Standards

Standard compounds were obtained from three manufacturers, with Wako supplying caffeic acid, ferulic acid, protocatechuic acid, and *trans*-cinnamic acid. However, chlorogenic acid (3-caffeoylquinic acid) and *p*-coumaric acid were supplied from MP Biomedicals, Inc. (France) and shikimic acid was obtained from Kanto Chemical Co., Inc. (Japan).

RNA extraction and cloning of *EPSPS* gene fragment from cucumber

Nine leaf discs (10 mm diam, ca. 100 mg fresh weight) from the third leaf of cucumber were frozen in liquid nitrogen and stored at −80°C. RNA was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA samples were treated with DNase (DNA-free, Ambion, USA) and reverse transcription was performed using RETRO Script Kit (Ambion) as per manufacturer's instructions.

Using degenerate primers, a fragment from *EPSPS* gene homologues was PCR amplified and cloned from cucumber cDNA. Consensus sequence was derived from amino acid sequence homology among EPSPS proteins of *Conyza sumatrensis* (NCBI GenBank accession number AAY40473), *Fagus sylvatica* (ABA54869), *Camptotheca acuminate* (AAV64030), *Allium macrostemon* (ABE77393), *Dicliptera chinensis* (AAL27697), and *Eleusine indica* (CAD01096). Degenerate primers synthesised for the *EPSPS* gene were EPSPS-dF (5′-AGGGRACWACDGTNGTDGA-3′) and EPSPS-dR (5′-GTRCCWAGRRWACAHTCAAC-3′). This primer combination successfully amplified the expected 354 bp fragment using TaKaRa PCR Thermal Cycler (Takara, Japan), with 1.25 units of GeneTaq DNA polymerase (Nippon Gene, Japan), 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH9.0), 0.01% Tween-20, 2 mM $MgCl_2$, 125 µM of each dNTP, 0.4 µM of each primer and 100 ng of template cDNA in a final volume of 50 µl. Reaction conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 52°C for 90 s, 72°C for 1 min, with a final extension at 72°C for 10 min.

The *EPSPS* gene fragment was cloned into pGEM®-T Easy Vector System (Promega, USA) and sequenced using the ABI Prism 3100 Genetic Analyser (Applied Biosystems, USA), and the homology to corresponding genes from other plant species confirmed using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

The expression of the *EPSPS* gene

For the analysis of the gene expression, multiplex reverse transcription PCR (RT-PCR) was used to simultaneously amplify transcripts of the *EPSPS* gene and constitutively express the reference gene based on the translation elongation factor 1α (*ef1-α*) (Mahé et al. 1992). The primer sets for the RT-PCR were EPSPS-RTF (5′-ACTTGGGTTGGACGTTGAAG-3′), EPSPS-RTR (5′-ACCAAATCCCCAATTGGTCT-3′) for *EPSPS* gene, and EF-RTF (5′-ATTGTGGTCATTGGTCATGT-3′), and EF-RTR (5′-CCTATCTTGTAVACATCCTG-3′) for *ef1-α* gene. Gene expression was detected by fluorescent-based semiquantitative RT-PCR using forward primers labelled with the fluorophore, fluorescein isothiocyanate (FITC) at the 5′ end. Amplification products of RT-PCR were separated on a 1.5% agarose gel, visualised, and analysed using a Molecular Imager

PharosFX System (Bio-Rad Laboratories, USA) and the Quantity One (Bio-Rad Laboratories, USA) software. The ratio between *EPSPS* and *ef1-α* expression was determined and plotted as fold increases above values measured at 0 h for the DW U treatment. The experiment was repeated twice.

Effect of caffeic acid and Zn^{2+} on NADPH oxidation

Gene expression and activity of NADPH oxidase increased in ASM-treated cucumber plants prior to inoculation with the anthracnose fungus (Table 1; Deepak and Ishii 2006b). It was reported that NADPH oxidase activity was induced in Zn-deficient plants. Following the re-supply of Zn to deficient plants this phenomenon was reversed (Pinton et al. 1994). In the current study, the level of caffeic acid was increased at 12 h following ASM treatment in an experiment. The rapid increase in caffeic acid levels in the early stages of ASM-induced SAR in cucumber plants might have suggested the involvement of this phenolic in the regulation of reactive oxygen species (ROS) production. Therefore, the effects of caffeic acid and Zn^{2+} on NADPH oxidation were examined in this study.

Table 1 Induction of the expression of defence-related genes or activity of enzymes, and accumulation of SAR-associated compounds in cucumber plants following ASM application

Time after treatment (h)					
0	3	6	12	24	48
LOX[a]		LOX[a]			
	H_2O_2[b]	H_2O_2[b]			
		NOX[1]	*NOX*, NOX[1a]		
		SOD[2]		SOD[2a]	
		CAT[2a]		CAT[a]	
		LPO[2]	*LPO*		
			POX[3]	*POX*	*POX*
	CALS[2]	*CALS*	*CALS*	*CALS*	
			Shikimic acid***		
			EPSPS		
			PAL1[3]	*PAL1*	*PAL1*
			PR1-1a[3]	*PR1-1a*	*PR1-1a*
			CHIT[4]	*CHIT*	*CHIT*
			GLU[4]	*GLU*	*GLU*

The results shown above indicate upregulation of gene expression or enzyme activity and accumulation of compounds upon ASM treatment of the first true leaves of cucumber plants followed by challenge inoculation with *Colletotrichum orbiculare*. The underlined gene, enzyme and compounds were examined in the current study.

CALS callose synthase; *CAT* catalase; *CHIT* chitinase; *EPSPS* 5-enolpyruvylshikimate-3-phosphate synthase; *GLU* β-1, 3-glucanase; H_2O_2 hydrogen peroxide; *LOX* lipoxygenase; *LPO* lignin peroxidase; *NOX* NADPH oxidase; *PAL1* phenylalanine ammonialyase 1; *POX* peroxidase; *PR1-1a* pathogenesis-related protein 1-1a; *SOD* Cu/Zn superoxide dismutase

[a] Enzyme activity, [b] Quantity; [1] Deepak and Ishii (2006b); [2] Deepak et al. (2006); [3] Cools and Ishii (2002); [4] Narusaka et al. (1999)

Fresh third leaves were excised and homogenised in extraction buffer (50 mM HEPES-KOH; pH7.8, 250 mM sucrose, and 0.1 mM EDTA). The homogenate was filtered through four layers of absorbent gauze and the filtrate was centrifuged at 600 *g* for 15 min at 4°C. To obtain microsomes, the 600 *g* supernatant was centrifuged at 42,000 *g* for 20 min at 4°C and the resultant supernatant pelleted at 140,000 *g* for 1 h at 4°C. The final pellet was suspended in the extraction buffer (Shen et al. 2000). To determine the NADPH oxidation rate, an aliquot (200 µl) of the 600 *g* supernatant, microsomal fraction (42,000 *g* supernatant) or 140,000 *g* supernatant was added to a reaction mixture consisting of 50 mM HEPES-KOH (pH7.8), 0.1 mM EDTA, and 1 µM KCN in a final volume of 1 ml. The enzyme sample was pre-incubated for 3 min at 25°C. The reactions were then initiated by the addition of 100 µM NADPH. The NADPH oxidation rate was measured by the decrease of A_{340} after incubation for 15 min with a Viento multi-spectrophotometer (Dainippon Pharmaceutical Co., Ltd., Japan). The effect of caffeic acid and Zn^{2+} on NADPH oxidation activity was measured *in vitro* by treating the microsomal fraction with different concentrations of caffeic acid (0 µM, 15.6 µM, 31.3 µM, 62.5 µM, 125 µM, 250 µM, 500 µM) and $ZnCl_2$ (0 µM, 0.125 µM, 0.25 µM, 0.5 µM, 1 mM). The enzyme samples for Zn^{2+} experiments, aliquots of the 42,000 *g* supernatant were added along with 250 µM of caffeic acid and pelleted at 140,000 *g* for 1 h at 4°C. The caffeic acid-treated microsomal fraction was resuspended in extraction buffer, added to concentrations of $ZnCl_2$ and the suspensions were then centrifuged at 140,000 *g* for an additional 1 h at 4°C. Concentrations of the proteins were then determined using the method described by Bradford (1976).

Statistics

When the tendency of the results from each independent experiments was the same, as found in most experiments, the average values of the experiments were combined and statistically analysed. Significant differences between the plots were examined using Two-Way ANOVA and Duncan's Multiple Range Test, performed with Axum 6.0 software (MathSoft, Inc., USA) and Excel Toukei version 5.0 software (Esumi Co., Ltd., Japan), respectively. Data presented were mean values from independent experiments.

Results

Detection of hydrogen peroxide in xylem fluids

After ASM treatment, the amounts of H_2O_2 started to increase in the xylem fluids (Fig. 1). Three hours after treatment, cucumber plants were challenge-inoculated with the anthracnose fungus. ASM I-treated plants showed significantly higher ($P<0.01$) amounts of H_2O_2 compared to those of the other treatments (Table 1; Fig. 1). The amounts of H_2O_2 from ASM U and DW I were also significantly higher than those of DW U ($P<0.01$) at 3 h after treatment but there were no differences between the ASM U and the DW I treatments. Six hours after treatment, the amounts of H_2O_2 from the ASM I treatment were significantly ($P<0.05$) higher than those from the other treatments. Indeed until 6 h after treatment, the amounts of H_2O_2 in xylem fluids were always higher in ASM I-treated cucumber plants.

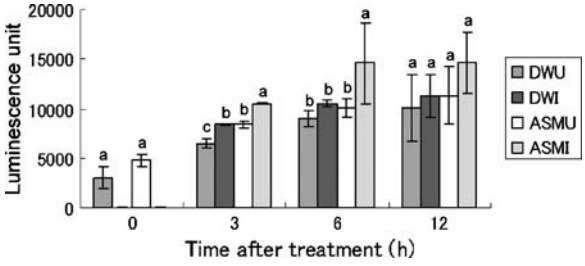

Fig. 1 Determination of hydrogen peroxide in the xylem fluids exuded from stumps of de-topped cucumber plants. Measurements were done using chemiluminescence in a ferricyanide-catalysed oxidation of luminol. Data presented are mean values from four independent experiments. *Bars* represent standard error of the mean. *ASM I* ASM-treated & inoculated; *ASM U* ASM-treated & uninoculated; *DW I* distilled water-treated & inoculated; *DW U* distilled water-treated & uninoculated. Cucumber plants were treated with ASM or DW at 0 h. Challenge inoculation with *C. orbiculare* was performed at 3 h after treatment. Means followed by the same letter within each sampling time point were not significantly different at the 5% level according to Duncan's Multiple Range Test

Interestingly, the accumulation of H_2O_2 increased with time in comparison with 0 h after treatment (Fig. 1). This result might have been due to the experimental environment (incubation of cucumber plants in a 100% humidity moist chamber for 24 h). However, the effect of the ASM treatment on the accumulation of H_2O_2 in xylem fluids of cucumber plants at each time point was comparable to that of the DW U treatment at the same time point.

Lipoxygenase assay

The optimum pH for LOX activity using linoleic acid as a substrate was 8.5 (Fig. 2a). Activity of LOX increased highly significantly ($P<0.01$) immediately after the application of ASM (Table 1; Fig. 2b). In addition, there was a significant ($P<0.05$) transient increase in LOX activity 6 h after treatment and inoculation with *C. orbiculare*, the anthracnose fungus, but no difference was found between the DW I and ASM I treatment categories in the activity of this enzyme.

Quantification of key components in shikimate, phenylpropanoid, and lignin biosynthetic pathways

In order to obtain more information about the mechanisms of ASM-induced SAR in cucumber, a number of key metabolites in the shikimate, phenylpropanoid, and lignin biosynthetic pathways were quantified in the current study.

The level of shikimic acid was highly significantly ($P<0.01$) lower in anthracnose fungus-inoculated cucumber plants (Fig. 3) than that of uninoculated plants 3 h post-treatment. At 12 h after treatment, the level of shikimic acid was significantly ($P<0.05$) higher in ASM U and DW I-treated plants than that found in DW U-treated plants, but there was no significant difference between the ASM U and DW I-treated plants. Shikimic acid levels also increased (Table 1; Fig. 3) highly significantly ($P<0.01$) in ASM I-treated plants. No other significant increase was detected in shikimate quantities extracted from ASM-treated plants at other sampling time points.

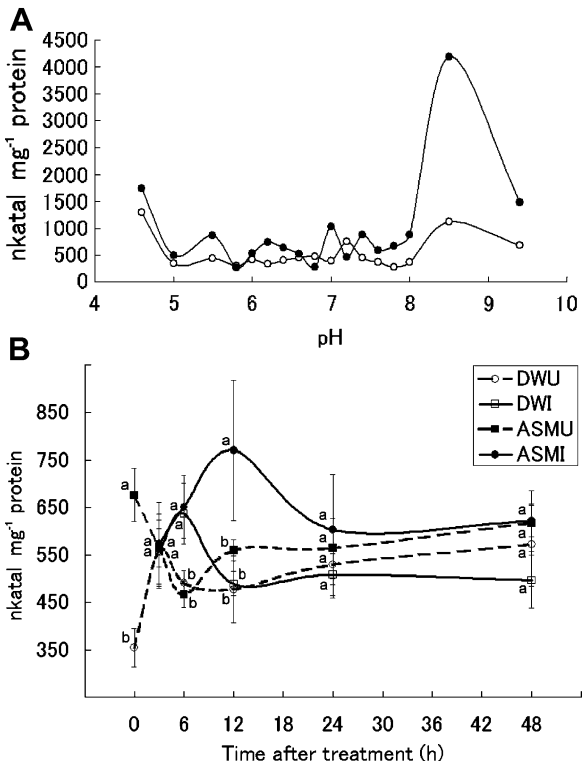

Fig. 2 Effect of pH on lipoxygenase (LOX) activity in cucumber leaf extracts using linoleic acid as the substrate. Extracts of crude enzyme were obtained from DW I (*open circle*) and ASM I-treated (*filled circle*) cucumber plants (**a**). LOX activity in extracts from DW- or ASM-treated cucumber plants with or without challenge inoculation with the anthracnose fungus. Values are the means from four independent experiments. *Bars* represent standard error of the mean (**b**). *ASM I* ASM-treated & inoculated; *ASM U* ASM-treated & uninoculated; *DW I* distilled water-treated & inoculated; *DW U* distilled water-treated & uninoculated. Cucumber plants were treated with ASM or DW at 0 h. Challenge inoculation with *C. orbiculare* was performed at 3 h after treatment. Means followed by the same letter within each sampling time point were not significantly different at the 5% level according to Duncan's Multiple Range Test

Phenolic compounds, among these *p*-coumaric acid and ferulic acid, were abundant in cucumber leaf extracts but the levels of these two compounds were not further increased by the ASM treatment (data not shown). However, the amount of caffeic acid was higher in ASM I-treated plants at 12 h post-treatment (data not shown). Levels of other phenolics (chlorogenic acid, protocatechuic acid, and *trans*-cinnamic acid) examined in this study did not increase appreciably upon ASM treatment (data not shown).

ASM induced SAR against anthracnose disease on cucumber plants. The disease lesions on ASM-treated plants were fewer and smaller than those on DW-treated plants. In the process of quantifying phenolics, BHA, an antioxidant was added to the extraction buffer to prevent quinine formation. The quantities of the two additional HPLC peaks at retention times of 48 min and 65 min were 1.24 and 3.07-folds, respectively, and were higher in ASM I-treated plants than obtained from the DW I-treated plants 7 days post-inoculation with *C. orbiculare*. Data from MS/MS analysis revealed (data not shown) that these HPLC peaks corresponded to oxidised derivatives of BHA. This evidence, though indirect, suggested that ASM treatment enhanced the accumulation of oxidised products, further reinforcing earlier evidence in cucumber tissues for increases in ROS levels (Park et al. 2002; Ishii et al. 2004) following ASM treatment.

Cloning and gene expression analysis of *EPSPS*

The gene expression of *EPSPS* was maintained at basal level in tissues from all treatment categories throughout the course of this study, with the exception of ASM I-treated plants at 12 h after treatment (Table 1; Fig. 4). The expression of the *EPSPS* gene had a 1.5-fold increase in ASM I-treated cucumber plants; however, this level of increase was not significantly different from those of other treatments at the $P<0.05$ level (Fig. 4).

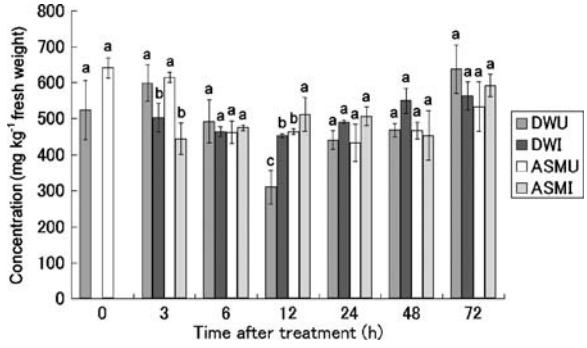

Fig. 3 Spectrophotometric determination of shikimic acid in cucumber plants after treatment with ASM followed by challenge inoculation with *C. orbiculare*. Data presented are mean values from three independent experiments. Bars represent standard error of the mean. *ASM I* ASM-treated & inoculated; *ASM U* ASM-treated & uninoculated; *DW I* distilled water-treated & inoculated; *DW U* distilled water-treated & uninoculated. Cucumber plants were treated with ASM or DW at 0 h. Challenge inoculation with *C. orbiculare* was performed at 3 h after treatment. Means followed by the same letter within each sampling time point were not significantly different at the 5% level according to Duncan's Multiple Range Test

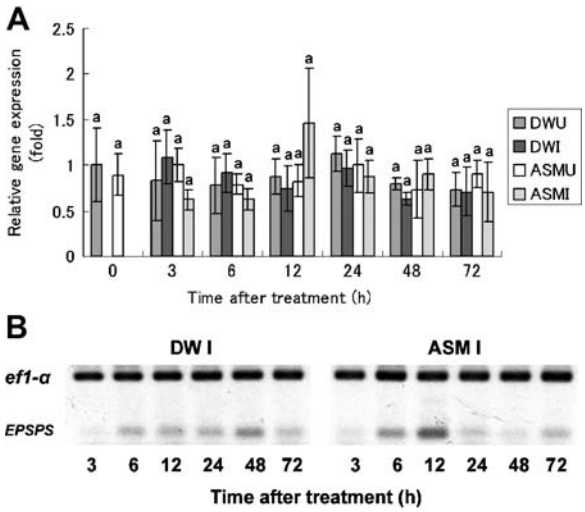

Fig. 4 Semiquantitative reverse transcription-polymerase chain reaction analysis of 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*) gene in third leaves of cucumber plants inoculated with *C. orbiculare* after ASM pre-treatment. Data presented are mean values from three independent experiments. *Bars* represent standard error of the mean (**a**). The size of the amplicon of *EPSPS* gene transcipts was 354 bp and the size of elongation factor 1α (*ef1-α*) gene used as a constitutive control was 709 bp. Gel shown is a representative of three independent replicates tested per treatment (**b**). The ratio of *EPSPS*/ *ef1-α* genes was determined and plotted as fold increases above values measured at 0 day. *ASM I* ASM-treated & inoculated; *ASM U* ASM-treated & uninoculated; *DW I* distilled water-treated & inoculated; *DW U* distilled water-treated & uninoculated. Cucumber plants were treated with either ASM or DW at 0 h. Challenge inoculation with *C. orbiculare* was performed at 3 h after treatment. Means followed by the same letter within each sampling time point were not significantly different at the 5% level according to Duncan's Multiple Range Test

Effect of caffeic acid and Zn^{2+} on NADPH oxidation

The effect of caffeic acid on NADPH oxidation rate was assayed and all the sub-cellular fractions (600 *g* supernatant, microsomal fraction, and the 140,000 *g* supernatant) tested showed an increase in the NADPH oxidation activity linked with the increase in caffeic acid concentration (Fig. 5). In both the total and microsomal fractions, NADPH oxidation activity was significantly increased by the ASM I treatment (Table 1; Deepak and Ishii 2006b); therefore, the microsomal fraction was further used and the effects of caffeic acid and Zn^{2+} on NADPH oxidation studied. After treatment of the microsomal fraction with $ZnCl_2$, the NADPH oxidation, which increased with caffeic acid, declined in amounts to a level similar to that in the untreated blank control (Fig. 6).

Discussion

Application of ASM to the first true leaves of cucumber plants 3 h prior to challenge inoculation with *C. orbiculare* successfully induced SAR against the anthracnose fungus. These results indicate the likelihood of the rapid translocation of a putative signal molecule from elicited lower leaves to the untreated upper leaves (Ishii et al. 1999). In this study, H_2O_2 in the xylem fluids increased initially in ASM-treated cucumber plants and increased even further 3 h after challenge inoculation with the anthracnose fungus. These results further confirm earlier findings (Cools and Ishii 2002; Deepak and Ishii 2006b) that the rapid accumulation of H_2O_2 in the xylem might relate to increases in levels of acidic peroxidase and NADPH oxidase gene expression upon ASM treatment. Consequently, H_2O_2 might modulate downstream signalling events including calcium mobilisation, protein phosphorylation and gene expression (Shetty et al. 2008). The phosphorylation of mitogen-activated protein kinase (MAPK) cascade activated by H_2O_2 has an important role in signal transduction. Perception of an extracellular signal activates a MAPK, which in turn can facilitate the translocation of the signal to the nucleus where it phosphorylates and activates transcription factors, thereby modulating gene expression (Shetty et al. 2008). The expression of genes of *GTPase Rac* 1- and 2-activated *MAPK* and transcription factors, especially *WRKY* as well as calcium-dependent protein kinase (*CDPK*) and genes encoding the protein secretory pathway, was enhanced within 12 h in ASM-treated cucumber plants (Deepak and Ishii 2006a, b). Furthermore, the expression of the *PAL* gene, primed by the ASM I treatment (Table 1; Cools and Ishii 2002), may also be related to the rapid accumulation of H_2O_2 from ASM-treated first true leaves to untreated upper leaves through xylem elements since the transcriptional activation of the *PAL* gene by H_2O_2 is well documented (Lamb and Dixon 1997).

Ishii et al. (2004) studied interactions between *C. orbiculare* and cucumber and detected H_2O_2 cytochemically within cucumber leaves. In that study, the frequency of penetration peg formation in ASM-treated plants was similar to that observed in plants treated with DW only. In contrast, infection hyphae were frequently observed in DW-treated plants but only rarely in those treated with ASM. Generation of

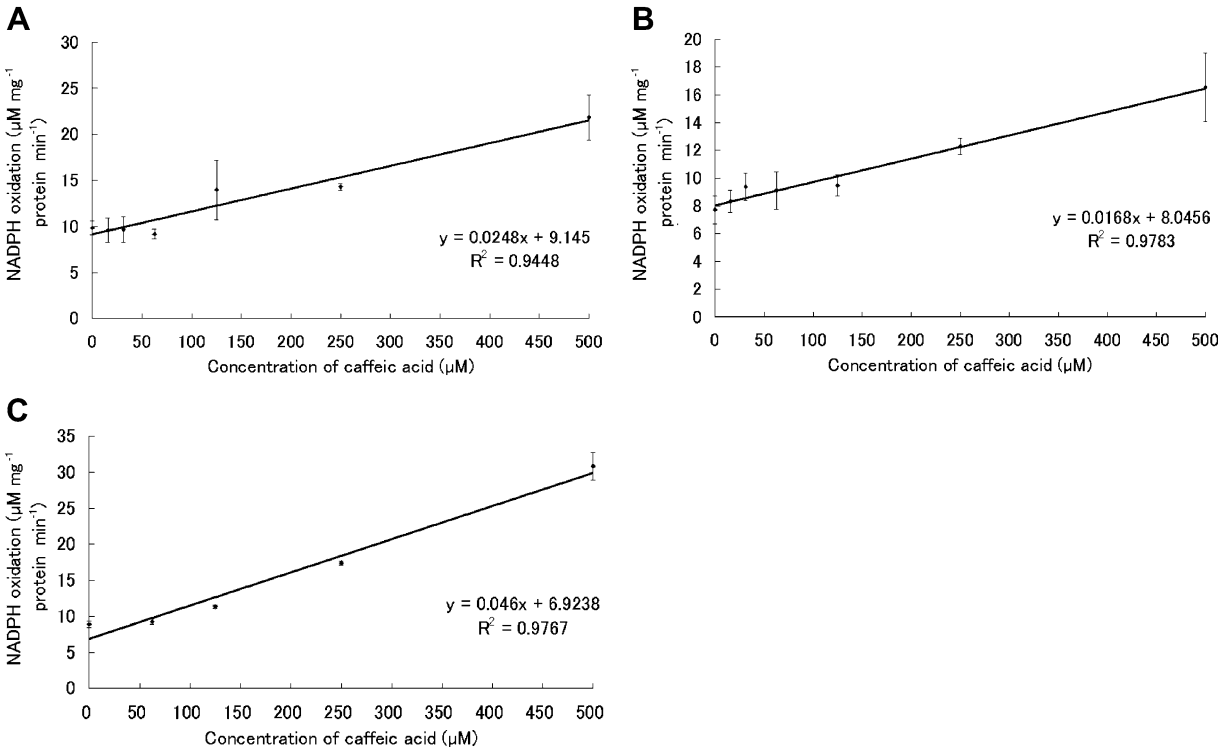

Fig. 5 Effect of caffeic acid on NADPH oxidation activity in sub-cellular fractions isolated from cucumber leaves. To determine NADPH oxidation rate, an aliquot (200 μl) of the 600 *g* supernatant (**a**), microsomal fraction (42,000 *g* supernatant, **b**) or 140,000 *g* supernatant (**c**) was added to a reaction mixture as described in "Materials and methods" with different concentrations of caffeic acid (0 μM, 15.6 μM, 31.3 μM, 62.5 μM, 125 μM, 250 μM, 500 μM) in a final volume of 1 ml. Bars represent standard error of the mean

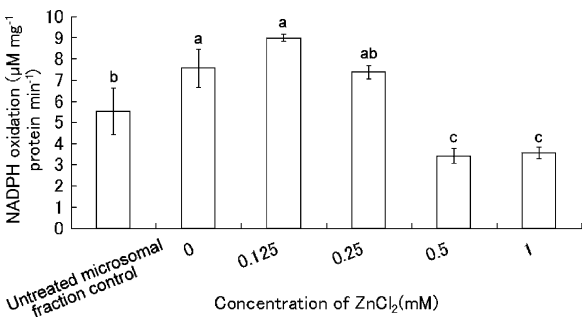

Fig. 6 Effect of $ZnCl_2$ on NADPH oxidation in caffeic acid (250 μM)-treated microsomal fractions isolated from cucumber leaves. The enzyme samples for Zn^{2+} experiments, aliquots of 42,000 *g* supernatant, were added with 250 μM of caffeic acid and pelleted at 140,000 *g* for 1 h. The pellets of microsomal fractions were resuspended into the extraction buffer (as described in "Materials and methods") added with different concentrations of $ZnCl_2$ (0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM) and the suspensions were centrifuged at 140,000 *g* for another 1 h. *Bars* represent the standard error of the mean. Means followed by the same letter within each sampling time point were not significantly different at the 5% level according to Duncan's Multiple Range Test

H_2O_2 was faster and greater in ASM-treated plants and appeared to be associated with a rapid accumulation of lignin within 24 h after application of ASM (Park et al. 2002), resulting in enhanced resistance of leaves against fungal penetration. Treatment with ASM did not appear to be accompanied by SA accumulation (Ishii et al. unpublished), whilst application of jasmonic acid (JA) increased susceptibility of cucumber to the anthracnose pathogen (Liu et al. 2008). Thus, neither SA nor JA is likely to function as a mobile inducer of SAR in ASM-treated cucumber plants. Earlier studies (Blee et al. 2004) indicated synergism between H_2O_2 and SA application regarding enhanced protection against *Pseudomonas syringae* pv. *tabaci* in tobacco plants. Hydrogen peroxide may be either the signal molecule itself or a by-product of the signal molecule translocated through the xylem elements of ASM-treated cucumber during SAR against the anthracnose pathogen. However, the results of the current study are not sufficient to demonstrate H_2O_2 as the mobile signal. Further

studies are required on the role of H_2O_2 in defence signalling. In addition, the involvement of phloem-mobile substances such as products of lipid metabolism, oligosaccharides, peptides and RNA species in ASM-induced SAR deserves examination in the cucumber-*C. orbiculare* pathosystem.

Exogenous Ca^{2+} has been reported to enhance LOX activity in cucumber exudates (Avdiushko et al. 1994). In addition, the Ca^{2+} pulse in xylem exudates of cucumber plants was rapidly induced following a foliar application of L(+)-adenosine (Ries et al. 1993). Therefore, the rapid increase in LOX activity observable within ASM-treated cucumber may be related to the transient exchange of Ca^{2+} between the apoplast and the cytoplasm of plant cells. Monitoring of the changes of different ions in the apoplast after ASM treatment should be the topic for a future study.

In this study, the amount of caffeic acid was higher at 12 h post-ASM I treatment. The accumulation of caffeic acid at this stage of ASM-induced SAR, possibly indicated that caffeic acid may be involved in the intial production of ROS. Lee (2005) reported that ROS was generated through the activation of NADPH oxidase in caffeic acid-treated HepG2 human hepatoma cells. In addition, the NAD(P)H oxidase activity, stimulated by caffeic acid, has also been investigated in microsomal fractions from cauliflower inflorescences (Askerlund et al. 1987). Furthermore, low amounts of Zn in plant cells may also induce O_2^--generating membrane-bound NADPH oxidase activity and following the re-supply of zinc to deficient plants it was also found to substantially decrease the rate of NAD(P)H oxidation and O_2^-production (Pinton et al. 1994). Alternatively, zinc could alter the oxidation-reduction potential of the NADPH-oxidising enzyme complex (Jeffery 1983). On the basis of these, we treated the three sub-cellular fractions (600 *g* supernatant, microsomal fraction, and the 140,000 *g* supernatant) with different concentrations of caffeic acid and obtained a dose-response relationship between NADPH oxidation and caffeic acid. The NADPH oxidation in the microsomal fraction increased with caffeic acid, but generally reverted to the basal level when subcellular fractions were treated with Zn. The substrate NADPH can be oxidised by NADPH oxidase and peroxidase. In this study, although NADPH oxidation was induced by caffeic acid, it was not inhibited by KCN, a well-known non-competitive peroxidase inhibitor, indicating that NADPH oxidase was involved. The mechanism of increase in NADPH oxidase activity by caffeic acid was unravelled only recently. Caffeic acid was reported as an antioxidant effective against free radicals (Castelluccio et al. 1995); evidence from the current study suggests that caffeic acid might chelate Zn^{2+} (Sommer et al. 2000) from plasma membranes and induce NADPH oxidation. Following replenishment of caffeic acid-treated microsomal fractions with Zn, however, the higher levels of NADPH oxidation induced by this phenolic were restored to normal, low basal levels.

The levels of *p*-coumaric and ferulic acids were abundant in cucumber leaf extracts. In addition, the expression of the lignin peroxidase (*LPO*) gene increased significantly at 6 h and 12 h in ASM I-treated plants (Deepak et al. 2006). Levels of *p*-coumarate and felulate were increased with such abundance along with caffeic acid in ASM I-treated plants that, as precursors of lignin, concomitant enhancement of *LPO* gene expression might have occurred. It is therefore reasonable to envisage lignin accumulation that would prevent tissue penetration by the anthracnose fungus 24 h after ASM I treatment (Park et al. 2002).

Increase in levels of shikimic acid is one of the events of SAR in plants. For example, shikimate levels rose to about two-fold in uninoculated upper (systemic) leaves of *Potato virus X*-infected potato plants compared to those within mock-inoculated control plants (Niehl et al. 2006). The gene *EPSPS* encodes an enzyme that catalyses a key step in the shikimate pathway giving rise to substrates feeding into the synthesis of lignins, flavanols and anthocyanins (Benfey et al. 1990). In the current study, ASM treatment and challenge inoculation with the anthracnose fungus were found to significantly increase shikimic acid concentrations at 12 h, coinciding with peak increases in the gene expression of *EPSPS* and *PAL* (Table 1; Cools and Ishii 2002). Thus shikimate accumulation might stimulate the expression of defence-related genes and further prime cucumber plants for resistance to infection by *C. orbiculare*.

In conclusion, the results of this study reinforce the case that H_2O_2 might be either the xylem-signal molecule itself or a by-product of the signal molecule generated during ASM-induced SAR in cucumber plants. The NADPH oxidase pathway was activated by ASM treatment (Deepak and Ishii 2006b) through

transient increases in levels of caffeic acid which might have induced the chelation of Zn from plasma membranes. The enhancement of NADPH oxidase activity triggered ROS production, in addition to upregulation of LOX activity, and orchestrated a systemic defence response in cucumber plants which actively prevented infection by the anthracnose fungus.

Acknowledgements We thank Drs. Y. Yogo, M. Ishizaka, S. Hiradate, S. A. Deepak, K. Baba and Y. Kobara, National Institute for Agro-Environmental Sciences for their technical guidance and Syngenta Japan Co., Ltd. for providing ASM. Special thanks are to Dr. J. M. Fountaine, Scottish Agricultural College, for the molecular technical help and useful suggestions during paper writing.

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