J Phytopathol 157:287–297 (2009)
doi: 10.1111/j.1439-0434.2008.01489.x

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INF1 Elicitin Activates Jasmonic Acid- and Ethylene-mediated Signalling Pathways and Induces Resistance to Bacterial Wilt Disease in Tomato

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Received April 26, 2008; accepted June 18, 2008

Keywords: elicitin, ethylene, induced resistance, jasmonic acid, *Ralstonia solanacearum*

Abstract

Phytophthora infestans, the cause of potato and tomato late blight disease, produces INF1 elicitin, a 10 kDa extracellular protein. INF1 induces a hypersensitive response (HR) and systemic acquired resistance in species of the *Nicotiana* genus and a few other genera. We analysed the response of tomato to INF1 and INF1*S3*, which has a Cys to Ser substitution at position 3 of the processed protein and therefore lacks HR induction activity in tobacco. No HR cell death was induced in either INF1- or INF1*S3*-treated tomato leaves. The expression of salicylic acid (SA)-responsive *PR-1a*(*P6*) and *PR-2a* genes was not induced by treatment with either INF1 or INF1*S3*. However, the expression of jasmonic acid (JA)-responsive *PR-6* encoding proteinase inhibitor II, *LeATL6* encoding ubiquitin ligase E3, and *LOX-E* encoding lipoxygenase, was up-regulated in tomato leaves treated with INF1 but not in those treated with INF1*S3*. Their induction was completely compromised in INF1-treated *jai1-1* mutant tomato, in which the JA signalling pathway is impaired. The accumulation of ethylene (ET) and the expression of ET-responsive genes were also induced in tomato by INF1 but not INF1*S3* treatment. The activation of JA and ET-mediated signals but not the SA-mediated signalling in INF1-treated tomato was also demonstrated by global gene expression analysis. INF1-treated tomatoes, but not those treated with INF1*S3*, exhibited resistance to bacterial wilt disease caused by *Ralstonia solanacearum*. Thus, INF1 seems to induce resistance to bacterial wilt disease in tomato and activate JA- and ET-mediated signalling pathways without development of HR cell death.

Introduction

Plants respond to pathogen infection by activating a complex plant immune system. This system is of two types: pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) and effecter-triggered immunity (ETI) (Jones and Dangl, 2006). PAMPs are the molecular signature common to many classes of pathogens and are recognized by pattern recognition receptors, thereby inducing a common set of defence responses. When a pathogen effector interferes with PTI, plants use nucleotide binding (NB)-leucine rich repeat (LRR) proteins to defend against pathogens and to activate ETI. In ETI, pathogen effectors, equivalent to pathogen virulence factors, are either directly or indirectly recognized as avirulence (*Avr*) factors by specific NB-LRR protein products encoded by resistance (*R*) genes on a gene-for-gene basis, and then the hypersensitive response (HR) accompanying cell death is generally induced. These two branches of the plant immune system share a common defence mechanism, although PAMPs-mediated signalling and NB-LRR-mediated signalling require some distinct components (Zipfel et al., 2004).

Elicitin, a small lipid binding protein secreted by the oomycetes *Phytophthora* and *Pythium*, induces HR cell death in a narrow range of plants, such as *Nicotiana* species in the Solanaceae and some radish and rape cultivars in the Brassicaceae (Ricci et al., 1989; Kamoun et al., 1993, 1997b; Panabieres et al., 1995; Keizer et al., 1998; Ponchet et al., 1999). All known elicitins share a conserved elicitin domain and are divided into five different classes based on their primary structure (Kamoun et al., 1997a; Jiang et al., 2005). Class I elicitins only contain the 10 kDa elicitin

domain and this class can be separated into acidic and basic isoforms: class IA and class IB. Highly acidic elicitins that possess a short hydrophilic C-terminal tail belong to class II. Class III elicitins possess the elicitin domain with a long (65-101) amino acid C-terminal *O*-glycosylated domain anchoring the protein to the cell wall to expose the N-terminal domain at the cell surface (Jentoft, 1990; Kamoun et al., 1997a). Cryptogeins are well-characterized elicitins isolated from *Phytophthora cryptogea*. Cryptogein A and B belong to class IA and IB elicitins, respectively, and induce an HR-like response, defence gene expression and systemic acquired resistance (SAR) to the black shank-causing agent *Phytophthora parasitica var. nicotianae* in tobacco (Keller et al., 1999). Class II cryptogein-ha1 and ha2 also induce an HR-like response in tobacco (Panabieres et al., 1995). Megaspermin elicitins, such as class I α- and β-megaspermins and class III γ-megaspermin, also activate a defence response with HR cell death in tobacco (Baillieul et al., 1995, 2003). Class Py contains an acidic elicitin of *Pythium vexans*, which appears on a distinct branch of the elicitin family phylogenic tree (Kamoun et al., 1997a).

INF1 is a well-characterized elicitin produced by the potato and tomato pathogen *Phytophthora infestans* (Kamoun et al., 1997b). The mature INF1 protein is generated by processing of the N-terminal signal peptide from pre-INF1 protein. INF1 belongs to acidic class IA elicitins and contains conserved elicitin motifs (Kamoun et al., 1997a; Ponchet et al., 1999). Since INF1 elicitin, as well as cryptogein and megaspermin, has elicitor activity for the HR in tobacco, it has been extensively used in studies of the HR and HR cell death in *Nicotiana* species (Kamoun et al., 1998, 1999; Sasabe et al., 2000; Peart et al., 2002; Kanzaki et al., 2003; Sharma et al., 2003; Yoshioka et al., 2003; Huitema et al., 2005; Takemoto et al., 2005). However, the response of *P. infestans* susceptible *Solanum* species to purified recombinant INF1 suggests that INF1 functions as a general elicitor in *Solanum* (Vleeshouwers et al., 2006).

Elicitins, which are structurally conserved in *Phytophthora* and *Pythium*, have an important function for the microbe as sterol scavengers and are expressed during host interaction (Kamoun et al., 1997b; Tyler, 2002; Jiang et al., 2005). Therefore, recently elicitins have been thought to function as oomycete PAMPs rather than being the elicitor specific for inducing the HR (Nurnberger et al., 2004). Actually, in elicitin-treated *Brassica rapa*, the expression of defence-related genes is activated with microscopic sectors of necrosis (Takemoto et al., 2005). Furthermore, two novel elicitins [oligandrin and CWP (cell wall protein fraction) possessing two major glycoproteins POD1 and POD2], which are produced by the non-pathogenic organism *Pythium oligandrum*, do not trigger HR cell death in tobacco but do induce jasmonic acid (JA)- and ethylene (ET)-responsive defence-related gene expression and resistance to late blight-causing *Phytophthora infestans* in tomato, the bacterial wilt-causing agent *Ralstonia solanacearum* in tomato and root-rot-causing agents *Aphanomyces cochlioides* and *Rhizoctonia solani* AG2-2 in sugar beet (Picard et al., 2000; Takenaka et al., 2003, 2006; Hase et al., 2006, 2008).

Salicylic acid (SA)-, JA- and ET-mediated signal transduction pathways play key roles in disease resistance to pathogens. In general, JA- and ET-mediated signalling is mainly required for resistance to necrotrophic pathogens, whereas resistance to biotrophic pathogens is mainly dependent on SA-mediated signalling (Glazebrook, 2005). Much evidence indicates that in tobacco treated with elicitin, the SA-mediated signalling pathway is activated. In INF1-treated tobacco exhibiting HR cell death, the expression of acidic chitinase and β-1,3-glucanase genes and rapid accumulation of H_2O_2 are induced (Sasabe et al., 2000; Huitema et al., 2005). Suppression of *rboh* gene expression, implicating active oxygen species including H_2O_2 for an oxidative burst, led to a reduction and delay of the HR cell death caused by INF1 in *Nicotiana benthamiana*, indicating that the oxidative burst is associated with INF1-induced HR cell death (Yoshioka et al., 2003). Although the activation of JA and ET signalling pathways in response to INF1 has not been well investigated, upregulation of basic β-1,3-glucanase gene expression has been confirmed using a transgenic tobacco line carrying a basic BGL2::GUS reporter construct (Huitema et al., 2005). In contrast to the characterization of the response of *Nicotiana* species to INF1, the response of other Solanaceae to INF1 elicitin has not been analysed in details. INF1 does not induce HR cell death in tomato at the same concentration needed for induction in tobacco (Kamoun et al., 1997b). However, if tomato can recognize INF1 elicitin as a PAMP, a certain set of defence responses may be activated in INF1-treated tomato.

In this study, to understand the response of tomato to INF1 elicitin, we analysed the expression of defence-related genes and enhanced resistance to *Ralstonia solanacearum*, a causal agent of bacterial wilt disease, in plants treated with INF1 and INF1*S3*, an inactive mutant in which the Cys at position 3 of the processed INF1 protein had been substituted by Ser (Kamoun et al., 1997b). INF1 elicitin activated JA and ET signalling pathways and suppressed bacterial wilt disease in tomato without the HR cell death.

Materials and Methods

Plant growth

Solanum esculentum cv. Micro-Tom, which is a laboratory-grown miniature tomato valuable as a functional genomics tool for plant pathology (Shibata, 2005), and homozygous mutant *jai1-1* of Micro-Tom (Li et al., 2004) were grown in quartz sand at 24°C in a growth chamber under continuous fluorescent light (9000 lux), fertilized with a 1000-fold-diluted Hyponex solution at 3-day intervals (Takahashi et al., 2005). Seedlings of *jai1-1/jai1-1* homozygotes were selected using a PCR-based assay described by Li et al. (2004). *Nicotiana benthamiana* was grown in soil at 28°C in the same growth chamber.

Preparation and infiltration of INF1 and CWP elicitins

Plasmids pFB53 and pFB52, respectively, constructed by cloning wild-type *INF1* ORF and mutated *INF1* ORF including a single amino acid substitution of Cys to Ser at position 3 of the processed INF1 protein into pFLAG-ATS (Sigma-Aldrich, New Haven, CT, USA), respectively, were used (Kamoun et al., 1997b). *Escherichia coli* BL21 containing pFB52 or pFB53 was incubated at 37°C in LB medium containing 0.4% glucose until the OD_{600} value of the cultures reached 0.6. To produce recombinant INF1 and INF1*S3* proteins fused with a FLAG peptide (FLAG-INF1 and FLAG-INF1*S3*) in *E. coli*, IPTG was added to the culture at a final concentration of 0.4 mM, which was further incubated at 37°C for 3 to 4 h. The cultures were centrifuged at 3000 ***g*** for 10 min and the supernatant obtained was dialysed against 0.01 M sodium phosphate buffer (pH 7.0) at 4°C for 12 h. The resulted solution containing the expressed FLAG-INF1 or FLAG-INF1*S3* protein was used for the following experiments. The amount of the expressed FLAG-INF1 and FLAG-INF1*S3* proteins in the dialysed solution was measured by comparison of the intensity of the bands of these proteins with those of purified FLAG-INF1 protein by immunoblotting using anti-FLAG antibody (Sigma-Aldrich). Purification of recombinant FLAG-INF1 and FLAG-INF1*S3* was performed using a FLAG M2 antibody affinity gel according to the instruction manual (Sigma-Aldrich). One millilitre of 100 nmol/ml FLAG-INF1 or FLAG-INF1*S3* protein was infiltrated into the leaves of 6-week-old *N. benthamiana* and 4-week-old tomato plants using a disposable 1 ml plastic syringe. The progression of cell death at the infiltration site of the leaves was observed 2 days after infiltration.

Preparation of CWP elicitin from the cell wall fraction of *Pythium oligandrum* isolate MMR2 was conducted by the method of Takenaka et al. (2006). CWP (0.5 mg/ml) was infiltrated and the results were observed as above for the recombinant proteins.

Analysis of defence gene expression

The expression of tomato pathogenesis-related (*PR*) genes encoding proteinase inhibitor II (*PR-6*), acidic and basic β-1,3-glucanase (*PR-2a* and *PR-2b*), acidic PR-1 protein [*PR-1a(P6)*] and three genes encoding lipoxygenase (*LOX-E*), JA-inducible ubiquitin ligase E3 (*LeATL6*) and ET-related transcription factor (*ERF2*) of tomato was analysed. Total RNA was isolated from the leaves of 4-week-old tomato (*S. esculentum* cv. Micro-Tom) by the acid guanidium-phenol-chloroform method (Chomczynski and Sacci, 1987). Fifteen micrograms of total RNA was loaded in each line of a 1.2% denaturing agarose gel. Northern hybridization was performed according to Sambrook and Russell (2001). To detect the expression of *PR-6*, *PR-2b*, *LOX-E*, *LeATL6* and *ERF2* genes, ~1000 bp fragments of cDNA of each gene described previously were used as probes for northern hybridization analysis (Hase et al., 2006, 2008; Hondo et al., 2007). The DNA fragments for detecting transcripts from *PR-1a(P6)*, *PR-2a* and *LOX-E* by northern hybridization were also amplified by reverse PCR with primers 5′-CATAACGATGCCCCGTG-CCCAAGTCGG-3′ and 5′-GTAAGGACGTTGTCCGATCCAGTTGCC-3′ for *PR-1a(P6)* (Van Kan et al., 1992), 5′-CAGGAGCGCAGCCTATCGGAGTATG-3′ and 5′-GGCCTCTGGTCAGGTTTAAAGAGTC-3′ for PR-2a (Van Kan et al., 1992) and 5′-AGACAAATCCTAATCAATGG-3′ and 5′-CAATAATAAGTTCTATATAG-3′ for *LOX-E* (National Centre for Biotechnology Information [NCBI] accession no. AY008278). First-strand cDNA as a template for reverse transcriptional PCR was reverse-transcripted from total RNA of tomato leaves using a ThermoScript RT-PCR kit (Life Technologies, Rockville, MD, USA). One microgram of first-strand cDNA was added to 50 μl of 10 mM Tris–HCl (pH 8.3) containing 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM each primer, and 5 units of Go-Taq DNA polymerase (Promega, Madison, WI, USA) for PCR. The reaction ran with the program 30 cycles at 94°C for 1 min, 55°C for 2 min, and 73°C for 1 min. The PCR product was purified and cloned into the *Eco*RV site of pBluescript SK + (Stratagene, La Jolla, CA, USA). To confirm that the expected DNA was cloned, the nucleotide sequence of each insert was determined by the Sanger method using an ABI model 310A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The amplified *PR-6*, *PR-1a*, *PR-2a(P6)*, *PR-2b, LeATL6, LOX-E* and *ERF2* DNA fragments were labelled with digoxigenin using a DNA labelling kit (Roche, Penzberg, Germany) and detected using the CDP-*Star* reagent (New England Biolabs, Beverly, MA, USA) following the instruction manuals.

As a control experiment for induction of SA-inducible gene expression, 0.5 mM SA was infiltrated into tomato leaves and RNA was isolated 24 h after infiltration for northern analysis.

Global gene expression analysis using a tomato microarray

To investigate the alteration in the gene expression pattern of INF1-treated tomato 12 h after treatment, global gene expression analysis using an Affymetrix GeneChip tomato genomic array (Affymetrix, Santa Clara, CA, USA) was conducted. In the tomato array, the 9200 non-redundant genes were composed. Total RNA was isolated from the leaves of six tomato plants treated with 100 nmol/ml FLAG-INF1 and distilled water as a control using an RNeasy plant mini kit (Qiagen, Hilden, Germany), respectively. Isolated total RNA were amplified and labeled as described in the GeneChip Expression Analysis Technical Manual, Rev.5 (Affymetrix). First, total RNA (1 μg) was converted into double-stranded cDNA using the One-Cycle cDNA Synthesis Kit (Affymetrix). Double-stranded cDNA was purified by using a GeneChip Sample Cleanup Module (Affymetrix). *In vitro* transcription reactions were performed using a GeneChip IVT Labeling Kit, which includes T7 RNA polymerase and Biotin-labelled ribonucleotides.

Biotin-labeled cRNA was purified using a GeneChip Sample Cleanup Module. The concentration of cRNA was calculated from light absorbance at 260 nm using a UV spectrophotometer. Next, cRNA (5 μg) was fragmented at 94°C in the presence of a fragmentation buffer (Affymetrix). Five micrograms of the cRNA was hybridized to Affymetrix GeneChip® Tomato Genome Array. The array was incubated for 16 h at 45°C, then automatically washed and stained with GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The Probe Array was scanned using a GeneChip Scanner 3000 7G. GeneChip Operating Software (Affymetrix, Inc.) software and further analysis was carried out with GeneSpring(R) 7.3.1 (Agilent Technologies, Palo Alto, CA, USA). Raw intensity values from each chip were normalized to the 50th percentile of the measurements. Each gene was normalized to that gene in the respective controls to enable comparison of relative changes in gene expression levels between treatment with 100 nM/ml FLAG-INF1 and distilled water control. Genes upregulated >3-fold or downregulated <-3-fold that of controls were listed.

Measurement of ethylene production

Each plant treated with INF1, INF1*S3* or CWP elicitin was grown in a gas-tight serum flask. At the time of ET measurement, 1 ml gas samples were withdrawn through the rubber seal. The concentration of ET was determined by gas chromatography as described by Hase et al. (2006). As a control, plants were treated with distilled water. The mean values of ET concentration and their SD were calculated for three plants in each experiment. ET production was determined by subtracting ET concentration in elicitin-treated plants from the background value in control plants. Each set of experiments was repeated two times.

Inoculation of *Ralstonia solanacearum* and disease assessment

Ralstonia solanacearum strain 8242 (race 1, biovar 4) was prepared as described by Nakaho et al. (2004). Fifty millilitre of 100 nmol/ml INF1, 100 nmol/ml INF*S3* or 0.5 mg/ml CWP was infiltrated into the middle region of a fully expanded leaf of a 4 week-old tomato plant. After incubation at 29°C, 7000 lux, for 24 h, 50 μl of a bacterial suspension (1×10^8 cfu/ml) was infiltrated into the top region of INF1-, INF1*S3*- or CWP-infiltrated leaves using a disposable 1 ml syringe. The plants were incubated at 29°C at 7000 lux for an additional 7 days and inspected daily for wilting symptoms. As a control, distilled water-infiltrated tomato leaves were inoculated with the same strain. Disease severity was rated as 0, no wilting; 1, 1–25% wilting; 2, 26–50% wilting; 3, 51–75% wilting; 4, 76–100% wilting or dead (Roberts et al., 1988). Each assay was repeated in three successive trials. Within each experiment, eight plants were inoculated.

For measurement of bacterial growth, eight plants were harvested at 7 days after inoculation. Hundred milligrams of the leaves was homogenized with 0.1 ml of distilled water. The homogenate was diluted with distilled water at 10- to 10^4-fold for counting bacterial colonies. Appropriate dilutions were plated on CPG agar medium containing 0.1% of yeast extract and 0.001% of tetrazolium chloride (Hendrick and Sequeira, 1984). Plates were incubated at 28°C for 2 days, and then the bacterial colonies were counted. Bacterial counts were expressed as colony-forming units per mg leaf tissue.

Results

Response of tomato and *N. benthamiana* to INF1

INF1 or INF1*S3*, with the Cys at position 3 of the processed INF1 protein substituted to Ser as a control, was infiltrated into the leaves of tomato and *N. benthamiana*. Although necrosis appeared at the infiltration site of INF1-treated but not INF1*S3*-treated *N. benthamiana* at 2 days after infiltration, neither 100 nmol/ml INF1 nor INF1*S3* infiltration which is a usual elicitin concentration inducing the HR in tobacco, induced necrosis in tomato leaves (Fig. 1). Necrosis did not appear in 300 nmol/ml INF1-infiltrated tomato (cv. Micro-Tom) leaves even 7 days after infiltration (data not shown). Furthermore, in INF1-treated tomato leaves, microscopic necrosis was not observed by trypan blue staining (data not shown).

Expression of SA-responsive genes in tomato treated with INF1

In order to determine whether INF1 activates the SA-mediated signalling pathway in tomato as well in INF1-treated tobacco, expression of two SA-responsive tomato *PR* genes, tomato *PR-1a(P6)* and *PR-2a*, which correspond to tobacco *PR-1a(P6)* and *PR-2a*, was analysed by northern hybridization at 6, 12 and 24 h after treatment. Transcripts of neither tomato *PR-1a(P6)* nor *PR-2a* were detected in either INF1- or INF1*S3*-infiltrated tomato leaves at 24 h after treatment, whereas their expression was induced by

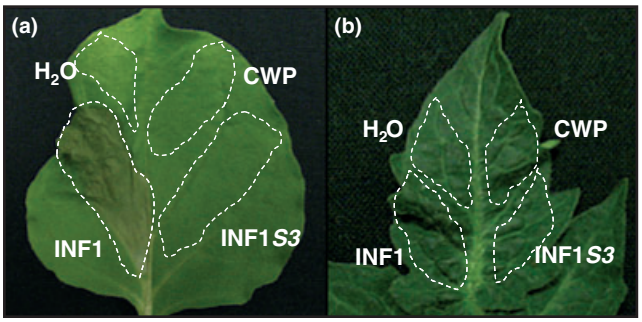

Fig. 1 Response of tomato and *Nicotiana benthamiana* to INF1 and INF1*S3* elicitins. (a) 100 nmol/ml INF1 and INF1*S3* were infiltrated into fully expanded leaves of *N. benthamiana*. (b) 100 nmol/ml INF1 and INF1*S3* were infiltrated into fully expanded leaves of tomato (*Solanum esculentum* cv Micro-Tom). CWP elicitin of *Pythium oligandrum* and distilled water (H_2O) were also infiltrated as controls. Infiltrated regions were indicated by dashed lines. At 2 days after infiltration, the leaves were photographed

exogenous application of SA (Fig. 2). Induction of the SA-responsive *PR* gene expression was not also observed either 6 or 12 h after treatment (data not shown). These results indicate that SA signalling may be not activated in either INF1- or INF1*S3*-infiltrated tomato leaves.

Activation of JA- and ET-mediated signal transduction pathways in tomato treated with INF1

To determine whether INF1 induces defence responses in tomato leaves despite the absence of either HR cell death or activation of SA-mediated signalling pathway, we analysed the expression of defence-related genes at the infiltration site of INF1 and INF1*S3*-treated tomato leaves. Expression of JA-responsive *PR-6*, encoding proteinase inhibitor II, and *LOX-E*, encoding lipoxygenase (*13-LOX*), was clearly induced in INF1- but not INF1*S3*-treated tomato leaves (Fig. 3). *LeATL6*, encoding ubiquitin ligase E3, also was upregulated in INF1- but not INF1*S3*-treated tomato leaves (Fig. 3). As shown in Fig. 3, the level of induction of these three genes in INF1-treated tomato leaves was similar to that in CWP elicitin-treated tomato leaves, which we previously showed to induce the activation of JA signalling without HR cell death (Hase et al., 2006; Takenaka et al., 2006; Hondo et al., 2007). Furthermore, earlier induction of *LeATL6* than *PR-6* and *LOX-E* expression in INF1-treated tomato leaves was also consistent with the timing of expression of these three JA-responsive genes observed in CWP elicitin-treated leaves.

To further confirm the activation of the JA-mediated signalling pathway in INF1-treated tomato leaves, expression of *PR-6* and *LOX-E* was analysed in INF1- and INF1*S3*-treated *jai1-1* mutant tomato in which JA signalling is impaired (Li et al., 2004). Upregulation of *PR-6* and *LOX-E* expression was compromised in INF1-infiltrated *jai1-1* mutant tomato (Fig. 4). These results suggest that the JA-mediated signalling pathway is activated in INF1-treated tomato.

Synergistic activation of ET-mediated signalling with JA-mediated signalling is generally observed in the defence response to necrotrophic pathogens (Glazebrook, 2005). To investigate the activation of ET signalling in INF1 and INF1*S3*-treated wild-type tomato, expression of ET-responsive *PR-2b,* encoding basic β-1,3-glucanase, and *ERF2*, encoding ET responsive transcription factor, was analysed. In INF1-infiltrated leaves but not in those infiltrated with INF1*S3*, expression of *PR-2b* and *ERF2* was induced as in CWP-infiltrated tomato leaves (Fig. 5). Furthermore, the amount of ET significantly increased in INF1-infiltrated tomato plants but not in INF1*S3*-infiltrated plants (Fig. 6). Thus, not only JA signalling but also

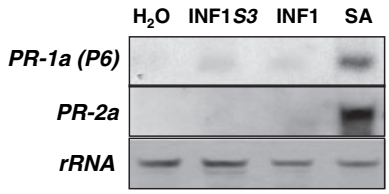

Fig. 2 Expression of SA-responsive defence-related genes in INF1- and INF1*S3*-infiltrated tomato leaves. Expression of *PR-1a(P6)*, encoding pathogenesis-related 1 protein, and *PR-2a*, encoding acidic β-1,3-glucanase, in INF1- and INF1*S3*-infiltrated tomato leaves was analysed by northern hybridization 24 h after infiltration. As controls, expression of these two genes in 0.5 mM SA and distilled water (H_2O)-infiltrated tomato leaves was done by northern analysis. rRNA was detected as an internal control

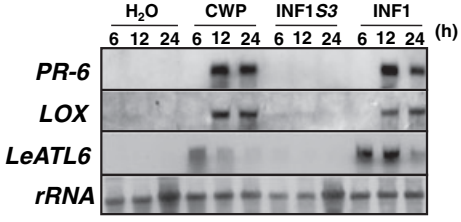

Fig. 3 Expression of JA-responsive defence related genes in INF1- and INF1*S3*-infiltrated tomato leaves. Expression of *PR-6*, encoding proteinase inhibitor II, *LOX-E*, encoding plastid lipoxygenase, and *LeATL6*, encoding ubiquitin ligase E3, in INF1- and INF1*S3*-infiltrated tomato leaves was analysed by northern hybridization at 6, 12 and 24 h after infiltration. As controls, expression of these three genes in CWP elicitin of *Pythium oligandrum* and distilled water (H_2O)-infiltrated tomato leaves was done by northern analysis. rRNA was detected as an internal control

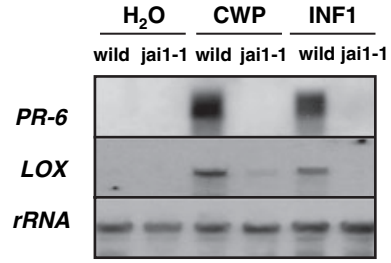

Fig. 4 Expression of JA-responsive defence-related genes in INF1-infiltrated wild-type and *jai1-1* mutant tomato leaves. Expression of *PR-6*, encoding proteinase inhibitor II, and *LOX-E*, encoding plastid lipoxygenase, in INF1-infiltrated wild-type and *jai1-1* mutant tomato leaves was analysed by northern hybridization at 12 h after infiltration. As controls, expression of these three genes in CWP elicitin of *Pythium oligandrum* and distilled water (H_2O)-infiltrated tomato leaves was done by northern analysis. rRNA was detected as an internal control

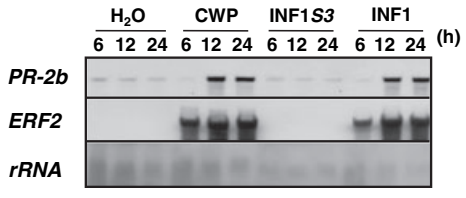

Fig. 5 Expression of ET-responsive defence-related genes in INF1- and INF1*S3*-infiltrated tomato leaves. Expression of *PR-2b*, encoding basic β-1,3-glucanase and *ERF2*, encoding ET-responsive transcription factor, in INF1- and INF1*S3*-infiltrated tomato leaves was analysed by northern hybridization at 6, 12 and 24 h after infiltration. As controls, expression of these two genes in CWP elicitin of *Pythium oligandrum* and distilled water (H_2O)-infiltrated tomato leaves was done by northern analysis. rRNA was detected as an internal control

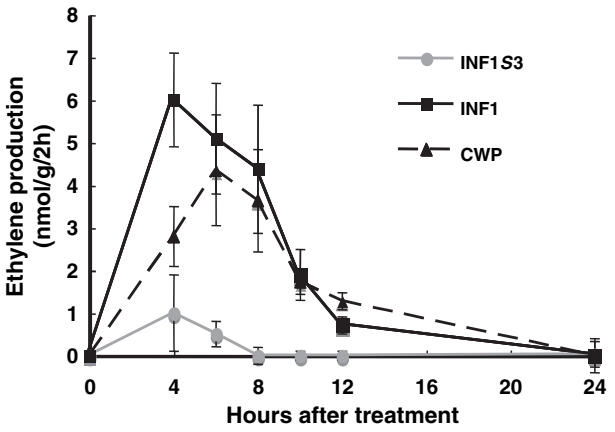

Fig. 6 Accumulation of ET in tomato leaves infiltrated with INF1 and INF1*S3*. The amount of ET in INF1 (squares), INF1*S3* (circles) and CWP (triangles)-infiltrated tomato plants relative to non-infiltrated tomato plants is shown. The average ET accumulation for three infiltrated plants in each treatment was measured and the bars indicate the resulting SD

ET signalling was activated by INF1 and activation was dependent upon the Cys at position 3 of the processed INF1 protein.

Global gene expression analysis in INF1-treated tomato leaves

To further understand the response of tomato leaves to INF1 at the molecular level, we compared the global gene expression pattern of INF1-treated tomato with a distilled water-treated control using an Affymetrix GeneChip tomato genome array composing the 9200 non-redundant genes. Microarray analysis of RNA isolated from INF1-treated tomato leaves and the control at 12 h after treatment indicated that 66 genes were upregulated more than threefold (Table 1) and 68 genes were downregulated to less than 1/3-fold (Table 2) in response to INF1. Two pathogenesis-related genes: *PR-2b* encoding basic β-1,3-glucanase (3.574-fold) and *PR-6* encoding proteinase inhibitor II (5.184-fold), *ERF2* encoding ethylene response factor 2 (3.042-fold), *LeATL6* encoding ubiquitin ligase E3 (3.219-fold) and lipoxygenase (5.691-fold) gene which were upregulated in INF1-treated tomato leaves by northern hybridization analysis (Figs 3 and 5) were included in 66 genes upregulated more than threefold by microarray analysis (Table 1). This fact strongly indicates that this microarray data should be reliable to monitor global gene expression in INF1-treated tomato leaves. Except for these genes, other JA and ET-related genes, ex. genes encoding an ET receptor homolog, an ET responsive transcription factor and allene oxide synthase involving in JA biosynthesis, were also upregulated in INF1-treated tomato leaves (Table 1). Many genes encoding enzymes involved in lipid metabolism were also listed among the upregulated genes (Table 1). However, expression of SA-responsive *PR* genes, for example, *PR-1a(P6)* and *2a*, was less than twofold induction (data not shown). The altered gene expression pattern indicated that JA- and ET-mediated signalling pathways but not the SA signalling pathway are activated in INF1-treated tomato leaves without development of HR cell death.

Enhanced resistance to *Ralstonia solanacearum* in tomato plants treated with INF1

To investigate enhanced disease resistance to pathogens in INF1-treated tomato in which the JA- and ET-mediated signal transduction pathways had been activated, the development of bacterial wilt symptoms caused by *R. solanacearum* and bacterial growth were studied in INF1- and INF1*S3*-infiltrated tomato plants. As CWP elicitin can induce enhanced resistance to *R. solanacearum* in tomato without HR cell death but with activation of JA and ET-mediated signalling pathways (Hase et al., 2006, 2008), CWP-treated tomato plants were used as a positive control. As shown in Fig. 7a,b, bacterial wilt was significantly suppressed in INF1- as well as CWP-infiltrated tomato. However, in INF1*S3*-infiltrated tomato, wilt symptoms developed at the same severity in the distilled water-infiltrated control. In INF1-infiltrated tomato, bacterial growth was significantly suppressed as well as in CWP-infiltrated tomato (Fig. 7c), while bacterial growth in INF1*S3*-infiltrated tomato reached a similar level in the distilled water-treated control. These results indicated that enhanced resistance to *R. solanacearum* was dependent upon the Cys at position 3 of the processed INF1 protein as well the activation of the JA and ET signalling pathways was dependent on it.

Discussion

In tomato (*S. esculentium* cv. Micro-Tom), 100 nmol/ml INF1 which is a usual elicitin concentration inducing the HR in tobacco, activated JA and ET signalling pathways without HR cell death. The activation of the SA signalling pathway and SAR against a broad range of pathogens in INF1-treated tobacco accompanied by HR cell death has been well characterized. However, the activation of the JA and ET signalling pathways in response to INF1 has not been well investigated except for upregulation of basic β-1,3-glucanase gene expression in an INF1-treated transgenic tobacco (Huitema et al., 2005). In cryptogein-treated tobacco showing HR cell death, the expression of both SA-responsive acidic *PR* genes and JA-responsive basic *PR* genes is induced (Keller et al., 1996a; b). Treatment of tobacco with γ-megaspermin also induces the accumulation of acidic and basic PR-1, PR-2, PR-3 and PR-5 proteins and SAR (Cordelier et al., 2003). Thus, cryptogein and megaspermin may be able to activate not only SA signalling but also JA and ET signalling in tobacco. However, SAR was compromised in cryptogein and γ-megaspermin-treated *nahG* tobacco expressing a SA degrading salicylate hydroxylase, thereby preventing SA accumulation and upregulation of acidic *PR* genes (Keller et al., 1996b; Cordelier et al., 2003). Therefore, in cryptogein and γ-megaspermin-treated tobacco showing HR cell death,

Table 1
Genes with upregulated expression in INF1-treated tomato leaves

Transcript ID	Locus	Gene title[a]	Fold-induction[b]
Les.129.1	AF317515.1	divinyl ether synthase	7.067
LesAffx.66953.1	AW651552	peroxidase precursor	6.991
Les.51.1	X79337.1	ribonuclease	6.964
LesAffx.8850.1	AW034398	subtilisin-like protease	6.863
LesAffx.51285.1	AI895802	esterase/lipase/thioesterase domain containing protein	6.611
LesAffx.8720.1	BI921446	serine hydroxymethyltransferase	6.334
Les.54.1	AW218809	PR-1a1 protein (ethylene inducible)	6.204
LesAffx.3455.2	CN385030	putative beta-expansin 2 precursor-like protein	6.117
Les.764.1	BI929955	purine and other phosphorylases, family 1 protein	5.707
LesAffx.56301.1	AI895341	lipoxygenase, LH2 domain containing protein	5.691
Les.764.2	BG626572	nucleoside phosphorylase	5.554
LesAffx.12670.3	BF113197	UAA transporter family	5.348
LesAffx.15953.1	AW033344	DC1 domain containing protein	5.325
Les.3741.1	AF146691.1	Eli3 protein	5.221
Les.4022.1	X94946.1	proteinase inhibitor II	5.184
LesAffx.62617.1	BM409727	transferase family protein	5.165
Les.4222.1	AY034149.1	alternative oxidase 1b	5.117
Les.4307.1	AY257487.1	basic PR5-like protein	5.102
Les.28.2	AF092654.1	NRT2;1 protein	5.067
LesAffx.65156.1	AI895164	fatty acid desaturase	5.005
Les.3619.1	J04099.1	proteinase inhibitor I	4.786
Les.4317.1	AW625684	asparagine synthetase	4.775
LesAffx.52437.1	BI925563	membrane transport protein	4.753
Les.3490.1	U41103.1	ethylene receptor homolog	4.704
Les.4026.1	AY155579.1	xyloglucan-specific fungal endoglucanase inhibitor protein precursor	4.531
LesAffx.70534.1	AW030757	2OG-Fe(II) oxygenase superfamily protein	4.393
Les.13.1	AJ271093.1	allene oxide synthase	4.133
LesAffx.735.1	AW217158	putative DNA binding protein ABF1	4.081
Les.3502.1	AF332960.1	auxin-regulated dual-specificity cytosolic kinase	3.997
LesAffx.22491.2	CK715470	elicitor-inducible cytochrome P450	3.984
LesAffx.59668.1	BI208638	mitochondrial carrier protein	3.924
LesAffx.67017.1	AW032581	putative KNOX family class 2 homeodomain protein	3.924
LesAffx.37222.1	AI491001	GTPase-activator protein	3.879
Les.4150.1	AB017134.1	mitochondrial small heat shock protein	3.871
LesAffx.15898.1	AW649847	UDP-glucuronosyl/UDP-glucosyltransferase family protein	3.807
LesAffx.54123.1	BE450055	glycosyl transferase, family 8 protein	3.805
Les.2874.2	AI485944	mitogen-activated protein kinase 2	3.769
LesAffx.9910.1	CN385590	WRKY transcription factor 71 (Transcription factor WRKY09)	3.762
Les.3766.1	U77719.1	ethylene-responsive late embryogenesis-like protein	3.717
Les.3581.1	U72396.1	class II small heat shock protein Le-HSP17.6	3.682
LesAffx.295.2	AI772896	Zn-finger, AN1-like domain containing protein	3.668
Les.2591.1	M80608.1	basic beta-1,3-glucanase	3.574
LesAffx.71016.1	BI923152	plant invertase/pectin methylesterase inhibitor domain containing protein	3.571
Les.1724.2	AI776156	glutathione S-transferase, C-terminal domain containing protein	3.571
Les.5916.1	AJ715788.1	anaerobic basic leucine zipper protein	3.569
LesAffx.67116.1	X94944.1	lipid desaturase-like protein	3.539
LesAffx.11992.1	BI206094	multifunctional transport intrinsic membrane protein 2	3.536
Les.3528.1	AF258810.1	aldehyde oxidase	3.516
LesAffx.68107.1	AI485479	Avr9/Cf-9 rapidly elicited protein 231	3.507
LesAffx.20391.1	BF176422	cyclin-like F-box domain containing protein	3.484
LesAffx.36193.1	AW034622	Zn-finger, C2H2 type domain containing protein	3.439
LesAffx.29757.2	AI898522	dioxygenase related to 2-nitropropane dioxygenase	3.436
LesAffx.42534.1	AW041123	similar to phosphatidic acid phosphatase type 2 domain containing 1A	3.411
Les.2476.1	BI208593	wound-induced protein	3.389
Les.5948.1	AY691331.1	MAPKK	3.384
LesAffx.11542.1	BI922302	calcium binding gene, DD112	3.355
Les.3132.1	X72732.1	(ERT 14) ripening-related mRNA	3.328
LesAffx.29757.2	AI898522	2-nitropropane dioxygenase (NPD)	3.323
LesAffx.71065.1	BM413117	pathogen-related protein similar to P16273	3.277
Les.4287.1	CB751564	transcription-associated recombination protein	3.275
LesAffx.62975.1	AW622674	RING-H2 zinc finger protein ATL6-like protein	3.219
Les.36.1	AF118843.1	ethylene receptor homolog (ETR4)	3.173
LesAffx.70722.1	AW030137	ETR1	3.128
LesAffx.50270.1	BI422773	gluconolactonase (carbohydrate transport and metabolism)	3.109
LesAffx.66096.2	BF097459	mevalonate kinase	3.089
Les.4102.1.S1	AY192368	ethylene response factor 2 (ERF2)	3.042

[a]Putative function of each gene product was determined by a BLASTN or BLASTX homology search using the TIGR tomato genome index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato) and GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).
[b]Fold-induction of each gene in the leaves at 12 h after INF1 and H_2O treatments was calculated by the expression value for INF1 divided by that of H_2O. When the gene expression value increased more than threefold in the INF1-treated plants, we identified the gene expression as altered reproducibly between two treatments.

Table 2
Genes with downregulated expression in INF1-treated tomato leaves

Transcript ID	Locus	Gene title[a]	Fold-suppression[b]
Les.99.1	X99452.1	extensin-like protein Dif54	−3.008
Les.4345.3	AI775226	pathogenesis-related protein P2	−3.011
Les.3237.1	BG625970	DnaJ-class molecular chaperone with C-terminal Zn finger domain	−3.021
Les.2377.1	BG125660	glycolate oxidase	−3.023
Les.190.1	L23424.1	phytoene synthase	−3.040
Les.2504.1	BG628687	SAM dependent carboxyl methyltransferase family protein	−3.044
Les.477.2	AW624887	sucrose transporter	−3.063
Les.3515.1	AF242849.1	IAA6 protein	−3.070
LesAffx.10650.1	AW036686	dormancy auxin associated family protein containing InterPro domain	−3.075
Les.4097.1	AF022018.1	IAA7 protein	−3.096
LesAffx.16952.2	AA824982	divalent cation tolerant protein CUTA	−3.105
Les.1816.1	AI777742	plastid and cyanobacterial ribosomal protein PSRP-3/Ycf65 family protein	−3.107
LesAffx.13252.2	BG124820	esterase precursor (EC 3.1.1.-) (early nodule-specific protein homolog)	−3.107
Les.5271.1	BT013613.1	HD-ZIP protein	−3.107
Les.3703.1	AF022020	IAA9 protein	−3.140
LesAffx.18117.1	AI775336	ribosomal protein L11	−3.197
LesAffx.55200.1	AW649455	lipolytic enzyme, G-D-S-L family protein	−3.201
Les.2696.1	BG128736	DNAJ domain protein	−3.205
Les.3047.1	BT013001.1	expansin 18	−3.208
Les.4187.1	BG628403	non-specific lipid transfer protein	−3.211
Les.3180.2	AI775197	rhodanese-like domain containing protein	−3.220
LesAffx.48402.1	CD002400	photosystem II reaction center protein PsbP family protein	−3.265
LesAffx.20903.1	AW932932	Bet v I allergen family protein	−3.270
Les.14.1	X98930	SBT2 protein	−3.298
Les.3299.2	BG626957	arginase 2	−3.303
LesAffx.70270.1	AI773931	glycosyl transferase, family 2 domain containing protein	−3.318
Les.4405.1	BG630861	protoporphyrin IX magnesium chelatase (EC 4.99.1.-)-like protein	−3.327
LesAffx.71228.1	AW038765	50S ribosomal protein L24, chloroplast precursor	−3.391
LesAffx.59441.1	BI205943	SPX, N-terminal domain containing protein	−3.395
Les.3065.2	BG630869	putative squamosa-promoter binding protein	−3.445
Les.61.1	Y10403.1	RNA-directed RNA polymerase	−3.510
Les.4457.1	AF035630.1	epidermal germacrene C synthase	−3.514
Les.406.1	BG629308	IQD11 (IQ-domain 11); calmodulin binding	−3.515
LesAffx.33890.1	BG134582	lipid binding START domain containing protein	−3.573
LesAffx.56734.1	AW032753	alpha/beta hydrolase fold domain containing protein	−3.577
Les.3974.1	X59282	metallocarboxypeptidase inhibitor	−3.606
Les.3174.1	BT012701.1	metallothionein II-like protein	−3.627
Les.3070.2	BG629293	Aaid phosphatase (Class B) family protein	−3.631
Les.3072.2	BM411575	mRNA binding protein precursor	−3.659
Les.263.1	M98466.1	polygalacturonase isoenzyme 1 beta subunit	−3.663
LesAffx.49476.1	BG123140	similar to interleukin-1 receptor-associated kinase 4	−3.720
Les.1614.1	BG628742	phospholipase C	−3.740
Les.1189.1	BG629967	short-chain dehydrogenase/reductase SDR family protein	−3.758
Les.173.1	AY725511.1	LePIP1 protein	−3.825
Les.1258.1	AY240230.1	gamma-aminobutyrate transaminase subunit precursor isozyme 2	−3.826
Les.3771.1	Y14387.1	lycopene epsilon-cyclase	−3.850
LesAffx.62264.2	AI489232	cytokine-like nuclear factor n-pac	−3.867
LesAffx.8440.1	BG126708	viral coat and capsid protein family protein	−3.943
LesAffx.22413.1	BI210086	plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor domain containing protein	−4.051
LesAffx.41330.1	CK574967	GUN4-like domain containing protein	−4.075
Les.314.2	AW217816	aquaporin 1	−4.127
Les.4072.1	AF242356.1	kinesin related protein	−4.128
LesAffx.26325.1	BG128147	Myb proto-oncogene protein (C-myb)	−4.142
Les.4428.3	BI935641	carbonic anhydrase	−4.243
Les.1617.2	AW934238	molecular chaperone Hsp90-2	−4.254
Les.3707.1	AF022013.1	IAA2 protein	−4.266
Les.3073.1	AJ785415	fruit-ripening protein	−4.270
Les.3234.1	BG735047	metallocarboxypeptidase inhibitor	−4.294
Les.3650.1	X55194	chalcone synthase	−4.335
Les.3035.1	BI423372	cathepsin D inhibitor protein	−4.344
Les.2012.1	BG627650	myo-inositol-1-phosphate synthase	−4.368
Les.2688.1	AJ560646.1	expansin 11	−4.469
Les.656.2	BE463153	ferredoxin domain containing protein	−4.479
LesAffx.37213.1	BG125438	basic helix-loop-helix dimerization region bHLH domain containing protein	−4.486
Les.4428.2	CK575032	carbonic anhydrase	−4.509
Les.1762	AI489730	protein phosphatase 2C family protein	−4.539
Les.3409.2	BF050508	fasciclin-like arabinogalactan protein 10 precursor	−4.548
LesAffx.17150.1	AW039265	ferredoxin-nitrite reductase, chloroplast precursor	−4.572

[a]Putative function of each gene product was determined by a BLASTN or BLASTX homology search using the TIGR tomato genome index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato) and GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).
[b]Fold-suppression of each gene in the leaves at 12 h after INF1 and H_2O treatments was calculated by the expression value for INF1 divided by that of H_2O. Ratios of <1 were transformed to a -1/ratio. Then, when the value of gene expression decreased less than –3-fold in the INF1-treated plant, we identified the gene expression as altered reproducibly between two treatments.

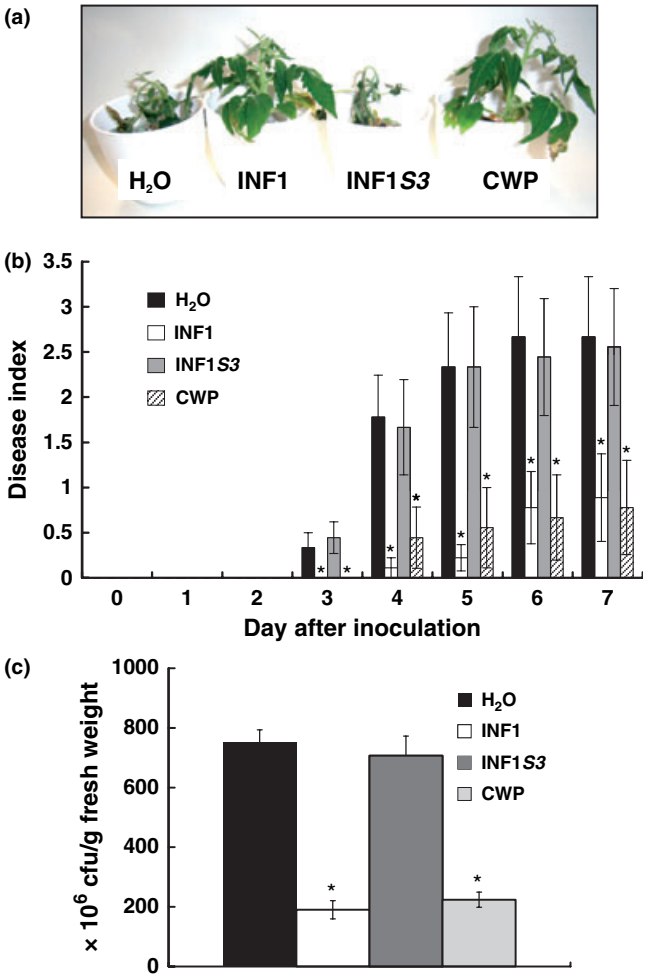

Fig. 7 Suppression of bacterial wilt disease caused by *Ralstonia solanacearum* in INF1-infiltrated tomato plants. INF1 and INF1*S3*, and as controls, distilled water (H_2O) and CWP elicitin of *Pythium oligandrum*, were infiltrated into tomato leaves. At 24 h after infiltration, plants were inoculated with 1×10^8 cfu/ml of *R. solanacearum*. At 5 days after inoculation, disease symptoms were observed. (a) Representative photographs of tomato plants inoculated with *R. solanacearum* 5 days after inoculation. (b) The severity of wilting symptoms in tomato plants at 5 days after inoculation. Eight plants were inoculated in each experiment. Values are the means of five independent experiments. (c) Bacterial growth in tomato plants at 7 days after inoculation. The growth of bacteria was shown as cfu per 1 g fresh weight leaf tissues inoculated with *R. solanacearum*. Eight plants were inoculated in each experiment. Values are the means of two independent experiments. In (b) and (c), an asterisk indicates a statistically significant difference compared with the control plants (H_2O) (Student's *t*-test; $P = 0.05$)

the SA signalling pathway seems to play a major role for SAR. Conversely, INF1 activated JA and ET signalling but not SA signalling in tomato. In INF1-treated tomato, enhanced resistance to *R. solanacearum* also developed. Thus, INF1 elicitin may induce resistance to bacterial wilt disease in tomato via activation of JA- and ET-mediated signalling pathways without HR cell death. There is not direct evidence that INF1-induced JA and ET responses are activated during infection with *P. infestans* in tomato. However, elicitins are structurally conserved in *Phytophthora* and *Pythium* and generally activate the defence system (Ponchet et al., 1999; Tyler, 2002). Recently, the activation of JA and ET signalling pathways by treatment with CWP elicitin and requirement of JA signalling for CWP-induced resistance to *R. solanacearum* were demonstrated at the interaction between non-pathogenic organism *P. oligandrum* and tomato plants (Hase et al., 2008). Furthermore, it has been demonstrated that JA signalling pathway were activated without the HR in non-host resistance of *Arabidopsis thaliana* to *P. infestans* (Huitema et al., 2003). Thus, in tomato infected with *P. infestans*, INF1 may be recognized as an oomycete PAMP, thereby triggering JA and ET-mediated basal defence system independent on the HR cell death. However, such basal defence response seems to be not enough to suppress significantly the growth of *P. infestans*, because consequently tomato is susceptible to *P. infestans*.

The molecular mechanism for recognition of INF1 in tomato and triggering of its downstream signalling pathways remains to be determined. Interestingly, the INF1-activated defence response in tomato was compromised by INF1*S3*, in which the Cys at position 3 of the processed INF1 protein was substituted by Ser. The Cys at position 3 determines the induction of the HR in tobacco (Kamoun et al., 1997b). Elicitins have sterol carrier activity (Blein et al., 2002). The core of elicitin is hydrophobic and the sterol molecule is entirely encapsulated in the core. INF1*S3* lacks one of the three disulfide bridges, thereby reducing sterol binding activity (Ricci et al., 1989; Kamoun et al., 1997b). The sterol binding ability of elicitin is correlated with HR induction activity and SAR in tobacco (Osman et al., 2001; Tyler, 2002). Therefore, the capability of INF1 to induce enhanced resistance to *R. solanacearum* and activation of JA and ET signalling in tomato may be also caused by its sterol binding ability.

The Cys at position 3 of the processed INF1 regulates the activation of defence response in both tomato and tobacco. Thus, the recognition step of the INF1 seems to be conserved between tomato and tobacco, but the earliest event of downstream signal transduction pathways for inducing defence response may be differentially activated. INF1 or an INF1-sterol complex seems to be recognized by elicitin-recognition receptor, which is postulated to be on the plasma membrane (Wendehenne et al., 1995). Thus, identification of the INF1-recognition receptor or receptor complex and its associated components in tomato and tobacco will clarify regulatory mechanism for INF1-triggered differential signal transduction pathway in two plant species.

In *N. benthamiana*, an oxidative burst, heat shock protein 90 (HSP90) and HSP70 molecular chaperones, and SGT1, a component of the Skp1/Cdc53/F-box protein ubiquitin ligase complex, are important for INF1-induced HR cell death (Peart et al., 2002; Kanzaki et al., 2003; Yoshioka et al., 2003; Huitema et al., 2005). Silencing of two mitogen-activated protein kinases (MAPKs), *WIPK* (wounding-induced protein kinase) and *SIPK* (SA-induced protein kinase),

reduces INF1-induced resistance to bacterial pathogens (Sharma et al., 2003). Furthermore, HR development in INF1-treated *N. benthamiana* leaves is suppressed by silencing of *NbMKK1*, which encodes a MAPK kinase physically interacting with SIPK (Takahashi et al., 2007). In cryptogein-treated tobacco, signalling events including calcium ion influx and activation of MAPKs also occur (Tavernier et al., 1995; Zhang et al., 1998). Thus, the study on the requirement of these signalling components for the INF1-triggered defence response in tomato will provide both a better understanding of INF1 downstream signalling conferring the INF1-induced defence response in plants and further insight for understanding the diversity of the PAMPs-triggered defence system.

Acknowledgements

We wish to thank Dr G.A. Howe, Michigan State University, USA for providing the *jai1-1* mutant tomato seeds, and Dr K. Nakaho, National Institute of Agro-Environmental Science, Japan for providing *Ralstonia solanacearum* strain 8242. This work was supported by a grant from the 'Research and Development Program for New Bio-industry Initiatives' of the Bio-oriented Technology Research Advancement Institute, Japan.

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