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RESEARCH PAPER

The involvement of jasmonates and ethylene in *Alternaria alternata* f. sp. *lycopersici* toxin-induced tomato cell death

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Abstract

Previous studies have shown that an ethylene (ET)-dependent pathway is involved in the cell death signalling triggered by *Alternaria alternata* f. sp. *lycopersici* (AAL) toxin in detached tomato (*Solanum lycopersicum*) leaves. In this study, the role of jasmonic acid (JA) signalling in programmed cell death (PCD) induced by AAL toxin was analysed using a *35S::prosystemin* transgenic line (*35S::prosys*), a JA-deficient mutant *spr2*, and a JA-insensitive mutant *jai1*. The results indicated that JA biosynthesis and signalling play a positive role in the AAL toxin-induced PCD process. In addition, treatment with the exogenous ET action inhibitor silver thiosulphate (STS) greatly suppressed necrotic lesions in *35S::prosys* leaves, although *35S::prosys* leaflets co-treated with AAL toxin and STS still have a significant high relative conductivity. Application of 1-aminocyclopropane-1-carboxylic acid (ACC) markedly enhanced the sensitivity of *spr2* and *jai1* mutants to the toxin. However, compared with AAL toxin treatment alone, exogenous application of JA to the ET-insensitive mutant *Never ripe* (*Nr*) did not alter AAL toxin-induced cell death. In addition, the reduced ET-mediated gene expression in *jai1* leaves was restored by co-treatment with ACC and AAL toxin. Furthermore, JA treatment restored the decreased expression of ET biosynthetic genes but not ET-responsive genes in the *Nr* mutant compared with the toxin treatment alone. Based on these results, it is proposed that both JA and ET promote the AAL toxin-induced cell death alone, and the JAI1 receptor-dependent JA pathway also acts upstream of ET biosynthesis in AAL toxin-triggered PCD.

Key words: AAL toxin, ethylene (ET), jasmonic acid (JA), PCD, tomato.

Introduction

Plants respond to a wide range of biotic and abiotic stresses by evoking reactions for resistance and preventing damage. Phytohormones play a central role in the signalling networks underlying the stress responses (Campos *et al.*, 2009). They act in a modular fashion and the action can be agonistic under some circumstances and antagonistic under others. Different hormones are involved in various stress responses, each leading to a specific set of downstream responses (O'Donnell *et al.*, 2003).

Jasmonic acid (JA) and ethylene (ET) are two of the major plant hormones involved in regulating plant defence responses (Anderson *et al.*, 2004; Melotto *et al.*, 2008). Under O_3 stress, ET is necessary for the development of lesions, whereas JA limits O_3-induced damage (Castagna *et al.*, 2007). The findings reported by O'Donnell *et al.* (2001, 2003) indicate that complete disease development of *Xanthomonas campestris* pv. *vesicatoria* on tomato requires host hormonal interactions among JA, ET, and salicylic

Abbreviations: AAL, Alternaria alternata f. sp. Lycopersici; ASC, Alternaria Stem Canker; ACC, 1-aminocyclopropane-1-carboxylic acid; CA, Castlemart; ET, ethylene; epi, epinastic; JA, jasmonic acid; JAI1, JASMONIC ACID INSENSITIVE1; jai1, jasmonic acid insensitive1; MeJA, methyl jasmonate; Nr, Never ripe; PCD, programmed cell death; qPCR, real-time quantitative PCR; 35S::prosys, 35S::prosystemin; STS, silver thiosulphate; $O_2^{\cdot -}$, superoxide anion; spr2, suppressor of (pro)systemin-mediated responses2.

acid (SA). JA and ET act as signalling molecules in resistance against or susceptibility to necrotrophic pathogen attack depending on the plant species and pathogens (Asai *et al.*, 2000; Devadas *et al.*, 2002; Anderson *et al.*, 2004; Egusa *et al.*, 2009; Onkokesung *et al.*, 2010). For instance, the Arabidopsis JA perception mutant *coi1*, but not JA biosynthesis mutants, exhibited a high level of resistance to a root-infecting fungus *Fusarium oxysporum*, suggesting that JA signalling mediated through COI1 (CORONATINE-INSENSITIVE1) in *Arabidopsis* is responsible for susceptibility to this pathogen (Thatcher *et al.*, 2009). There are reports of ET insensitivity leading to increased or decreased disease severity depending on the plant–pathogen combination (O'Donnell *et al.*, 2001). An intact JA–ET signalling pathway is thought to be necessary for the resistance of *Arabidopsis* to necrotrophic pathogens, such as *Botrytis cinerea* and *Erwinia carotovora* (Anderson *et al.*, 2004).

Alternaria alternata f. sp. *lycopersici* (AAL), a necrotrophic fungal pathogen, causes *Alternaria* stem canker on susceptible tomato (*Solanum lycopersicum*) cultivars and produces mycotoxins (Egusa *et al.*, 2009). AAL toxin is a host-specific pathogenicity factor of AAL-induced stem canker disease (Brandwagt *et al.*, 2002). AAL toxins and fumonisins produced by the unrelated fungus *Fusarium moniliforme* are members of a class of sphinganine analogue mycotoxins (SAMs) (Brandwagt *et al.*, 2002; Yamagishi *et al.*, 2006). SAMs structurally resemble sphinganine, an intermediate of sphingolipid ceramide biosynthesis (Mesbah *et al.*, 2000; Brandwagt *et al.*, 2002). *In vivo*, AAL toxins inhibit sphingosine *N*-acyltransferase (i.e. ceramide synthase), a key enzyme in the sphingolipid biosynthetic pathway (Westhuizen *et al.*, 1998). The disruption of sphingolipid biosynthesis causes marked accumulation of free sphingoid bases (Abbas *et al.*, 1994), which induce both programmed cell death (PCD) in susceptible plant cells and neoplastic events in mammals (Wang *et al.*, 1996; Morisseau *et al.*, 1999; Egusa *et al.*, 2009; Zélicourt *et al.*, 2009). Sensitivity to AAL toxin is not common in plants. It has host selectivity within different plant species (Abbas *et al.*, 1995; Mesbah *et al.*, 2000; Zélicourt *et al.*, 2009). Only a small number of *Solanaceous* species contain SAM-sensitive genotypes (Brandwagt *et al.*, 2002). Insensitivity to SAMs and fungal AAL is determined by the single co-dominant *Alternaria Stem Canker* (*ASC*) locus (Brandwagt *et al.*, 2000, 2002). The *Asc-1* gene is homologous to the yeast *Longevity Assurance Gene 1* (*LAG1*) (Brandwagt *et al.*, 2002; Zélicourt *et al.*, 2009), which is involved in sphingolipid biosynthesis and can prevent perturbation in sphingolipid metabolism and cell death to a large extent (Gechev *et al.*, 2004). Sensitivity to AAL toxin is limited to tomato genotypes homozygous for the recessive allele (*asc*/*asc*) associated with a mutation in the *Asc* gene (Morisseau *et al.*, 1999; Abbas *et al.*, 2008; Zélicourt *et al.*, 2009).

PCD is a genetically determined active suicide process involving a number of regulatory pathways, which ultimately lead to the selective removal of unwanted or severely damaged cells. It occurs at all stages of the plant life cycle and plays an important role in plants exposed to a broad range of biotic and abiotic stress (Orzaez *et al.*, 2001; Gechev *et al.*, 2004; Ma *et al.*, 2010). The cell death process involving AAL toxin and its susceptible tomato host is an excellent model for studying PCD in pathogen response pathways (Moore *et al.*, 1999; Asai et al., 2000). AAL toxin induces the disease symptoms of *Alternaria* stem canker (i.e. black necrotic spots between and along the veins, with a loss of turgour) on detached leaflets of susceptible tomato plants (Moore *et al.*, 1999; Mesbah *et al.*, 2000; Gechev *et al.*, 2004). The PCD process can be evaluated in this system in the absence of pathogen, which greatly simplifies the analysis (Moore *et al.*, 1999).

Plant mutants impaired in hormonal metabolism or signalling are commonly used to study the complex network of hormone pathways involved in host defence responses (Campos *et al.*, 2009; Egusa *et al.*, 2009). *Arabidopsis* mutants *fad3-2*/*7-2*/*8* (with reduced levels of trienoic fatty acids), *coi1* [insensitive to phytotoxin coronatine (COR) and JA], and *jar1* (with decreased sensitivity to JA) all exhibited enhanced susceptibility to *Pythium* spp., *Alternaria brassicicola*, *B. cinerea*, and *Plectosphaerella cucumerina* (Egusa *et al.*, 2009). Using the phytohormone mutants, fumonisin B_1 (FB_1)-induced cell death was found to require JA- and ET-dependent signal transduction pathways (Asai *et al.*, 2000). In tomato, JA-related mutants have also been characterized. For example, *jai1* (*jasmonic acid insensitive1*) contains a mutation in the tomato homologue of *Arabidopsis COI1* (Li *et al.*, 2004) and it cannot express JA-regulated genes in response to wounding and methyl jasmonate (MeJA). The *jai1* mutation is recessive and female sterile (Li *et al.*, 2004). In addition, a JA-deficient mutant *def1* (with a defective octadecanoid synthesis pathway) and a mutant *spr2* [a *suppressor of (pro)systemin-mediated responses2* mutation with reduced levels of trienoic fatty acids] have also been characterized (Howe *et al.*, 1996; Li *et al.*, 2003). In tomato, an ET-insensitive mutation *Never ripe* (*Nr*) caused by a single base substitution in the N-terminal coding region of the ethylene receptor gene *LE-ETR3* (i.e., *NR*) has been identified and it is homologous to *Arabidopsis ETR1* (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995). These mutants provide useful materials to investigate hormone functions in AAL toxin-induced PCD in tomato.

Previous reports have demonstrated that ET biosynthesis and ET perception via the NR receptor play a crucial role in AAL toxin-induced cell death signalling in detached tomato leaves (Moussatos *et al.*, 1994; Moore *et al.*, 1999). During the AAL toxin-induced cell death process in the *Arabidopsis loh2* mutant (a T-DNA knockout of a homologue of the tomato *Asc* gene), ET-responsive genes were among the ones to be up-regulated within seven hours (Gechev *et al.*, 2004). Knowledge of the role of plant hormones other than ET in AAL toxin-induced cell death is limited. Egusa *et al.* (2009) treated the tomato leaf discs of wild-type (WT) cv. Castlemart (CA) and *def1* with AAL toxin in the presence or absence of MeJA, and the results showed that endogenous JA biosynthesis and exogenous MeJA application did not affect the sensitivity of tomato to

AAL toxin. After *Arabidopsis* leaves were infiltrated with AAL toxin, JA marker genes were either not induced or down-regulated, and there was no indication of JA accumulation (Gechev *et al.*, 2004).

Although the action mechanism of ET in AAL toxin-induced tomato PCD has been elucidated, the role of JA in AAL toxin-induced PCD and the relationship to ET remain to be investigated. Here it is reported that JA signalling is involved in the PCD process using intact tomato leaflets of the JA-deficient mutant *spr2*, the *35S::prosystemin* transgenic line (*35S::prosys*, which accumulates high amounts of JA) (Howe and Ryan 1999), and the JA-insensitive mutant *jai1* combined with exogenous JA application. It was observed that JA actually plays a positive role in tomato sensitivity to the fungal toxin. Since both ET and JA signals play important roles in tomato sensitivity to AAL toxin, their interaction in the PCD process was investigated further by exogenous application of 1-aminocyclopropane-1-carboxylic acid (ACC) to the leaves of *spr2* and *jai1*, and silver thiosulphate (STS) to *35S::prosys*, as well as treatment of the *Nr* mutant with exogenous JA in the presence of AAL toxin.

Materials and methods

Plant materials and growth conditions

Tomato cultivar CA is the parental line for JA mutants *spr2*, *def1*, and *jai1* as well as the transgenic line *35S::prosys* (Supplementary Fig. S2 available at *JXB* online). Cultivar Pearson (PSN) is the parental line for the ET-insensitive *Nr* mutant. *35S::prosys* seeds were collected from a *35S::prosys* homozygote that had been backcrossed five times to its WT line cv. CA. They were included as controls where appropriate. The ET-overproducing mutant *epinastic* (*epi*), which is constitutively activated in a subset of ET responses (Fujino *et al.*, 1988; Barry *et al.*, 2001), and its WT line VFN8 were obtained from the Tomato Genetics Resource Center (University of California, Davis, CA, USA). Seeds were sown in seedling trays filled with a rich soil mixture after germination on filter paper. Seedlings were grown in a greenhouse, with temperatures ranging from 22°C to 28°C (night and day air temperature, respectively) and a 16 h photoperiod. Three weeks after germination, seedlings were transplanted to plastic pots (12 cm in diameter, 15 cm in depth) filled with perlite and turfy soil [3:1 (v/v)], which were watered daily and fertilized weekly with a half-strength Enshi nutrient solution (Yu and Komada, 1999). All experiments were carried out using fully expanded leaflets from nodes 4–6 (except for the terminal leaflets) of 7-week-old tomato plants.

Selection of jai1 *homozygotes*

jai1 homozygotes were screened according to Li *et al.* (2004). Briefly, surface-sterilized tomato seeds were germinated on filter paper until the roots were 2 cm in length. The germinated seedlings were treated with 1 mM MeJA (Sigma, St Louis, MO, USA). Approximately 24 h or 36 h later, MeJA-insensitive seedlings were selected by PCR using genomic DNA.

Detached leaflet bioassay

Treatment of tomato leaflets with AAL toxin was performed as described (Moore *et al.*, 1999; Spassieva *et al.*, 2002). Four excised leaflets from individual plants were incubated for 48 h on one filter paper in one Petri dish containing 4 ml of water or 0.2 μM AAL toxin under continuous light at 25°C (Supplementary Fig. S3 at *JXB* online). AAL toxin was a gift from Liangcheng Du (Department of Chemistry, University of Nebraska, Lincoln, NE, USA.).

Chemical treatment of different plant materials

To test the effect of exogenous JA on AAL toxin-induced PCD in the leaves of CA, *spr2*, PSN, and *Nr*, detached tomato leaflets were treated with solutions containing different concentrations of JA (0, 10, 100, or 500 μM) and 0.2 μM AAL toxin. As JA is an acid solution, to avoid being influenced by pH, different concentrations of sodium phosphate buffer (SPB, pH 7.0) were used as controls. JA at 500 μM was dissolved in 50 mM SPB (pH 7.0). Controls were treated with 0, 1, 10, or 50 mM SPB (pH 7.0) alone, respectively. ACC is an ET precursor, to test the effect of exogenous ET on AAL toxin-induced PCD in the leaves of *spr2* and *jai1*, solutions containing 0, 0.1, or 1 mM ACC (Sigma) and 0.2 μM AAL toxin were applied to the detached leaves. The inhibitor of ET action STS (0, 0.1, 1, and 2 mM) was applied to the leaflets of CA and *35S::prosys* in the presence of 0.2 μM AAL toxin. STS solutions were prepared by mixing $AgNO_3$ and sodium thiosulphate at a concentration ratio of 1:4. The concentrations referred to are those of the silver component (Bellés *et al.*, 1993). In all the experiments, unless otherwise stated, the detached leaflets were treated with distilled H_2O instead of 0.2 μM AAL toxin as controls. To assess the effect of exogenous chemicals on AAL toxin treatment, AAL toxin was dissolved in various chemical solutions.

Real-time quantitative PCR (qPCR) analysis

Tomato leaflets were sampled following AAL toxin treatment at different time points (0, 2, 6, 12, 24, and 36 h) and immediately immersed in liquid nitrogen. Total RNA extraction was carried out using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA was removed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA integrity was evaluated on a 1.5% (w/v) agarose gel. cDNA was synthesized using 5 μg of RNA with the RevertAid first-strand cDNA synthesis kit (Fermentas, Canada). cDNA was diluted in 100 μl of water and used as template for qPCR. See Table 1 for primer sequences.

qPCR was performed in a total volume of 25 μl, using 1 μl of diluted cDNA, 200 μM for each primer, and 12.5 μl of 2× SYBR Green PCR Master Mix (Takara, Japan) on an iCycler (Bio-Rad Inc., CA, USA). The qPCR program included a preliminary step of 30 s at 95°C, followed by 40 cycles of 95°C for 10 s and 58°C for 1 min. Tomato *actin* (GenBank accession number: AB199316) was used as an internal control (Table 1). Relative gene expression was calculated according to a $2^{-\Delta\Delta CT}$ method, in which $\Delta\Delta C_T=(C_{T,\ Target}-C_{T,\ actin})_{Time x}-(C_{T,\ Target}-C_{T,\ actin})_{Time0}$ (Livak *et al.*, 2001). Time*x* is any time point and time0 represents the C_T of non-treated control tissues. Three PCR replicates were conducted and the fold change in each target gene of time 0 was set to 1.

Callose assay and measurements of cell death

Leaves were cleared of pigment by vacuum-infiltrating alcoholic lactophenol followed by a 30 min incubation at 65°C. The leaves were then transferred to fresh alcoholic lactophenol solution and incubated overnight at room temperature. Cleared leaves were rinsed briefly in 50% ethanol, then in water, and stained with 0.01% aniline blue. Leaves were examined with a Zeiss Axiophot D-7082 fluorescence microscope with an excitation filter of 365±25 nm, a 400 nm dichroic mirror, and a 450 nm longpass emission filter (Underwood *et al.*, 2007). Cell death was evaluated using an electrolyte leakage assay. After sampling, tomato leaflets were immersed in ultrapure water for 30 min and the conductivity of the solution was measured with an LF-91 conductivity meter.

Table 1. Primer sequences used for real-time quantitative PCR (qPCR)

Gene	GenBank accession no.	Forward primer 5'–3'	Reverse primer 5'–3'	Product (bp)
actin	AB199316	TGGTCGGAATGGGACAGAAG	CTCAGTCAGGAGAACAGGGT	190
LoxD	U37840	GGCTTGCTTTACTCCTGGTC	AAATCAAAGCGCCAGTTCTT	72
PI-II	K03291	TGATGAACCCAAGGCAAATA	ACACAACTTGATGCCCACAT	154
ACO1	X58273	TTGCTCATTTCCTTTGTGGA	GGAAGCTAGCAAAGCAAACC	122
ACS2	X59139	ATCCACCTTGTTTGTGACGA	TGTTCATCGAGGATTTCAGC	86
ETR4	AF118843	CTGCAGATTGGAATGAATGG	ATAAGGCACCGTCAACATCA	123
NR	U38666	GCGGTTATGGTTCTGGTTCT	TGTCGAGCTACATCCAAAGC	194
ERF1	AY044236	ATTGGAGTTAGAAAGAGGCCAT	CTCATTGATAATGCGGCTTG	143

Then the samples were briefly autoclaved in the same solution and the conductivity measured again (total conductivity). The increase in electrolyte leakage (relative conductivity) is expressed as a percentage of the total (Gechev *et al.*, 2004).

Determination of $O_2\cdot^-$ production rate

The $O_2\cdot^-$ production rate was measured by analysing the nitrite formation from hydroxylamine in the presence of $O_2\cdot^-$. Frozen leaf segment was homogenized with 3 ml of 65 mM SPB (pH 7.8) and centrifuged at 5000 *g* for 10 min. The incubation mixture contained 0.9 ml of 65 mM SPB (pH 7.8), 0.1 ml of 10 mM hydroxylamine hydrochloride, and 1 ml of the supernatant. After incubation at 25°C for 20 min, 17 mM sulphanilamide and 7 mM α-naphthylamine were added to the incubation mixture. Ethyl ether in the same volume was added and centrifuged at 1500 *g* for 5 min. The absorbance in the aqueous solution was read at 530 nm (Zhou *et al.*, 2009).

Experimental design and statistical analysis

Data were analysed using Statistica (SAS Institute Inc., http://www.statsoft.com). Differences in relative conductivity (except for Fig. 2D) and the $O_2\cdot^-$ production rate in each figure were analysed by one-way analysis of variance (ANOVA); if the ANOVA analysis was significant (P <0.05), Duncan's multiple range test was used to detect significant differences between groups. In Fig. 2D the differences in relative conductivity between VFN8 and *epi* were analysed by Student's *t*-tests. Differences in gene expression in the mutant (*jai1* or *Nr*) treated with AAL toxin alone among different time points in each figure were analysed by one-way ANOVA; if the ANOVA analysis was significant (P <0.05), Duncan's multiple range test was used to detect significant differences between groups. Differences in gene expression of AAL toxin treatment alone between the mutant (*jai1* or *Nr*) and its parental line, as well as in gene expression of the mutant (*jai1* or *Nr*) between AAL toxin treatment alone and co-treatment with AAL toxin and exogenous hormone (ACC or JA) at each time point were analysed using Student's *t*-tests.

Results and Discussion

AAL toxin-induced PCD in tomato leaves is associated with endogenous JA levels and enhanced by exogenous JA application

The CA tomato cultivar was reported to be sensitive to the host-specific mycotoxin AAL toxin (Egusa *et al.*, 2009). In this study, the leaves of CA exhibited visible necrotic lesions at 36 h after treatment with 0.2 μM AAL toxin (data not shown), and typical necrotic lesions appeared at 48 h (Fig. 1).

JA has important roles in resistance against and susceptibility to pathogen attack. For example, enhanced resistance against the necrotrophic pathogen *B. cinerea* in the JA-insensitive *Arabidopsis* mutant *jin1* showed that JA is essential for susceptibility of *Arabidopsis* plants to this pathogen (Lorenzo *et al.*, 2004). JA has a promotional effect on susceptibility of tomato to the fungal pathogen AAL (Egusa *et al.*, 2009). To investigate whether endogenous JA levels are involved in tomato defence against AAL toxin, a tomato JA-deficient mutant *spr2* and a *35S::prosys* transgenic line were treated with AAL toxin. Compared with CA, development of necrotic lesions on *spr2* leaves was markedly decreased, while there was extensive cell death on *35S::prosys* leaves (Fig. 1A). Callose deposition is a typical early defence response. Superoxide anion ($O_2\cdot^-$) is one of the key reactive oxygen species (ROS). Relative conductivity is an indicator of plasma membrane damage. Callose deposition, $O_2\cdot^-$ production rate, and relative conductivity from leaflets were detected to measure further the extent of cell death. The leaflets of *spr2* exhibited a significant reduction in callose deposition compared with CA (Fig. 1B). The $O_2\cdot^-$ producition rate and the relative conductivity in *spr2* were also obviously lower than that in cv. CA (Fig. 1C, D). However, the callose deposition, $O_2\cdot^-$ levels, and relative conductivity in *35S::prosys* were markedly higher (Figs 1B, C, 4E). The results are consistent with the above cell death phenotypes.

The results showed that SPB (pH 7.0) application alone did not alter the relative conductivity in tomato leaves compared with H_2O treatment alone, and there were no significant differences between the toxin treatment alone and co-treatment with the toxin and buffer (data not shown). Besides, the relative conductivity in tomato leaves did not change significantly after treatment with different concentrations of STS, JA, or ACC compared with H_2O treatment alone (Supplementary Fig. S4 at *JXB* online). Thus, SPB, STS, JA, or ACC solutions were not toxic to detached tomato leaves. When CA and *spr2* leaves were treated with AAL toxin in the presence of different concentrations of JA, the result showed that 100 μM JA application caused a significant propagation of necrotic cell death in CA and *spr2* leaves (Fig. 1A), which was also reflected by the increased callose accumulation and $O_2\cdot^-$ levels (Fig. 1B, C). Accordingly, the relative conductivity of CA and *spr2* leaves increased to different extents after

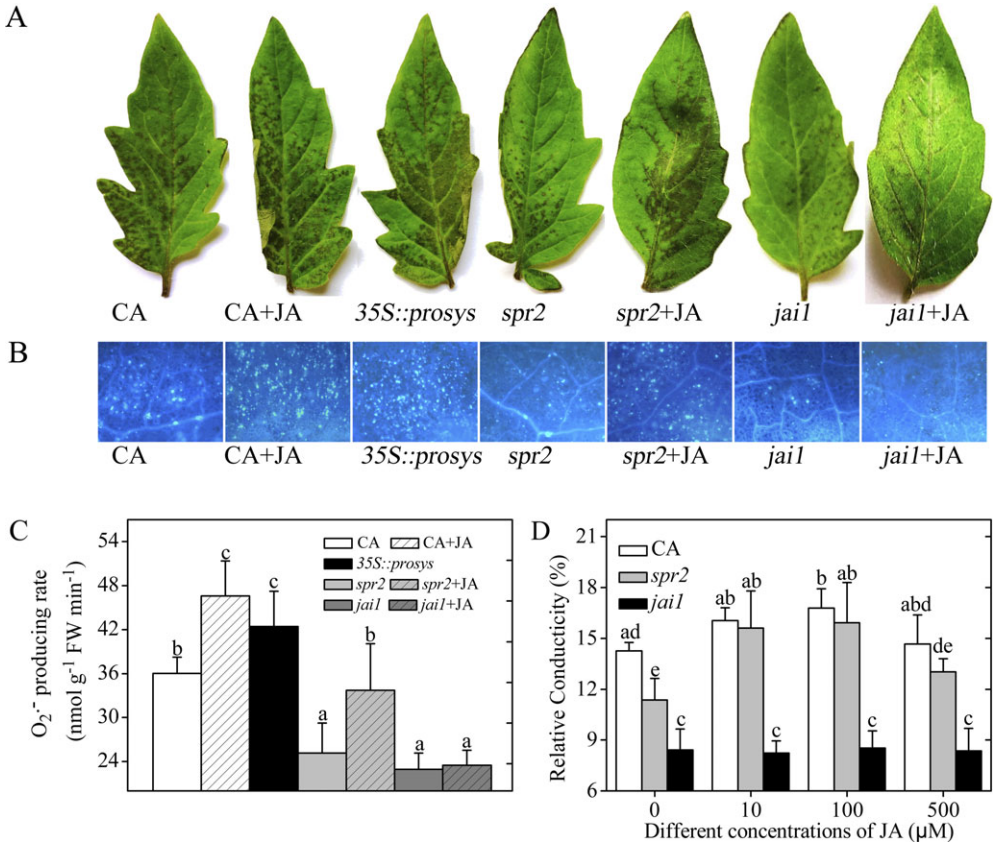

Fig. 1. Effects of JA levels on the sensitivity of detached tomato leaflets to AAL toxin. Detached leaflets were incubated under continuous light at 25°C for 48 h. (A) Symptoms of CA, *35S::prosys*, *spr2*, and *jai1* leaflets after 0.2 μM AAL toxin application or co-treatment with AAL toxin and 100 μM JA. (B) Callose deposition in tomato leaves in response to AAL toxin with or without 100 μM JA. Leaflets were stained with aniline blue to detect callose. Representative microscope images are shown. Callose deposits appear as bright spots on a dark background. Scale bars represent 100 μm. (C) Quantitative measurements of the $O_2{\cdot}^-$ level in tomato leaflets after AAL toxin application or co-treatment with the toxin and 100 μM JA. (D) Quantitative measurements of relative conductivity in tomato leaflets after AAL toxin application or co-treatment with the toxin and different concentrations of JA. Error bars indicate standard deviation. For each figure, letters indicate significant differences among treatments (P <0.05, Duncan's multiple range test).

10 μM and 100 μM JA application (Fig. 1D). The above results suggested that an impaired JA biosynthesis pathway attenuated the extent of tissue damage. However, higher endogenous JA levels in *35S::prosys* and exogenous JA application to CA increased the sensitivity to AAL toxin as compared with the isogenic WT parent CA (Fig. 1D, 0 mM JA bar; Fig. 4E, 0 mM STS bar) during this response.

The results are contradictory to the results reported by Egusa *et al.* (2009), who showed that reduced endogenous JA levels in *def1* and 100 μM MeJA treatment of CA and *def1* did not affect the sensitivity of the leaf discs to AAL toxin. This contradiction may be due to the use of intact leaflets in the present study versus the use of leaf discs. The experiments of Egusa *et al.* (2009) were repeated here and the same results as they reported were obtained (Supplementary Fig. S1 at *JXB* online). It is possible that compared with intact leaflets, the smaller leaf discs produced after severe mechanical wounding have a high background of diverse effects of JAs. In addition, JAs have several bioactive forms in plants. Compared with WT cv. CA, there may be free JA levels in *def1* as well as other conjugated forms of JA or analogues of JAs, which promote the AAL toxin-triggered cell death. Furthermore, after wounding for 1 h, the JA levels in *def1* and *spr2* were 20% and 6% of those of WT plants, respectively (C Li *et al.*, unpublished data). The product encoded by the *DEF1* gene is unknown (Howe *et al.*, 1996); however, the *SPR2* gene is known to encode a fatty acid desaturase (Li *et al.*, 2003). Therefore, it was decided to use the *spr2* mutant instead of *def1* to study the effect of endogenous JAs in the AAL toxin-induced PCD process.

Impaired perception of JA via the JAI1 receptor reduced the AAL toxin-induced cell death

The insensitivity of *coi1* mutants of *Arabidopsis* and tomato to COR, which is a phytotoxin produced by some strains of *Pseudomonas syringae* and exerts its virulence effects by activating the host's JA pathway, demonstrated that COI1 is required for the action of the toxin (Feys *et al.*, 1994;

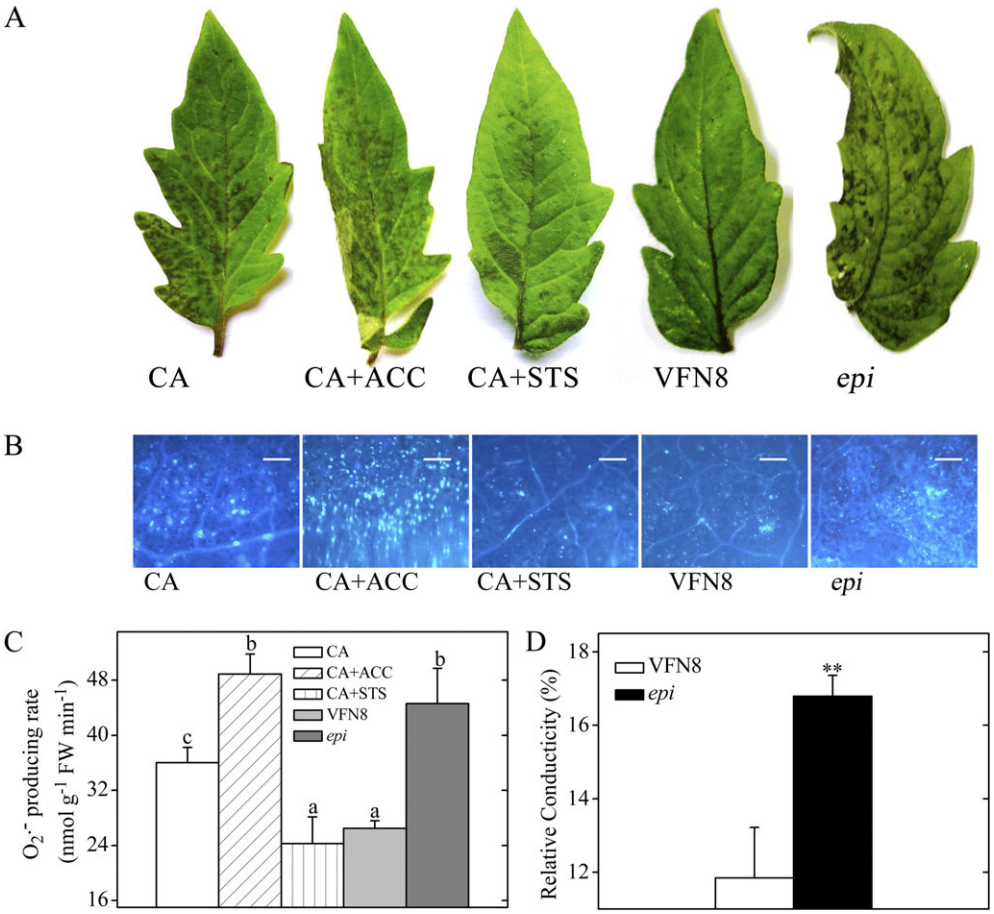

Fig. 2. Effects of ET levels on the sensitivity of detached tomato leaflets to AAL toxin. Detached leaflets were incubated under continuous light at 25°C for 48 h. (A) Symptoms of tomato leaflets after 0.2 μM AAL toxin application or co-treatment with the toxin and 0.1 mM ACC, an ET precursor, or 1 mM STS, an ET action inhibitor. (B) Callose deposition in tomato leaves in response to AAL toxin or co-treatment with the toxin and 0.1 mM ACC or 1 mM STS. Leaflets were stained with aniline blue to detect callose. Representative microscope images are shown. Callose deposits appear as bright spots on a dark background. Scale bars represent 100 μm. (C) Quantitative measurements of the $O_2^{\cdot-}$ level in tomato leaflets after AAL toxin application or co-treatment with the toxin and 0.1 mM ACC or 1 mM STS. Error bars indicate standard deviation. Letters indicate significant differences among treatments (P <0.05, Duncan's multiple range test). (D) Quantitative measurements of relative conductivity in leaflets of VFN8 and *epi* after AAL toxin application. Error bars indicate standard deviation. Asterisks indicate significant differences in *epi* leaves compared with VFN8 (**P <0.01; Student's t-test).

Zhao *et al.*, 2003). To elucidate further the role of JA signalling in the AAL toxin-elicited PCD process, the detached leaflets of the JA-insensitive mutant *jai1* (Li *et al.*, 2004) were treated with the toxin. Similarly to *spr2*, the toxin-treated *jai1* leaves displayed minor necrotic lesions at 48 h compared with CA (Fig. 1A), which was consistent with callose deposition, $O_2^{\cdot-}$ levels, and the relative conductivity in *jai1* and CA leaflets (Fig. 1B, C, D 0 mM bars). Exogenous JA application did not significantly alter the AAL toxin-induced cell death, callose deposition, $O_2^{\cdot-}$ levels, and relative conductivity in *jai1* leaves (Fig. 1A–D), which was different from the situation in *spr2*, suggesting that the perception of JA requires the JAI1 receptor in this PCD process. The above results indicate that tomato JAI1 has a crucial role in mediating the complex response of tomato leaves to AAL toxin. Based on these results, it can be concluded that a JA signalling pathway positively regulates the sensitivity of tomato leaflets to AAL toxin.

Exposure to O_3 leads to JA biosynthesis in *Arabidopsis* plants, and pre-treatment of O_3-sensitive *Arabidopsis* plants with exogenous MeJA alleviates O_3-induced PCD (Rao *et al.*, 2000; Rao and Davis, 2001). Asai *et al.* (2000) reported that FB_1-induced PCD is alleviated in the protoplasts of the *Arabidopsis* JA-insensitive mutant *jar1*, suggesting that the role of JA may be unique to certain stimuli.

ET biosynthesis and signalling through NR in tomato is responsible for sensitivity to AAL toxin-induced cell death

The essential role of ET in plant PCD is well known. For instance, O_3 induces ET biosynthetic gene expression, and treatment with the ET inhibitor aminoethoxyvinylglycine (AVG) decreases transcript abundances of these genes in *Arabidopsis* (Overmyer *et al.*, 2000; Tuominen *et al.*, 2004). FB_1-induced PCD is depressed in the *Arabidopsis* ET

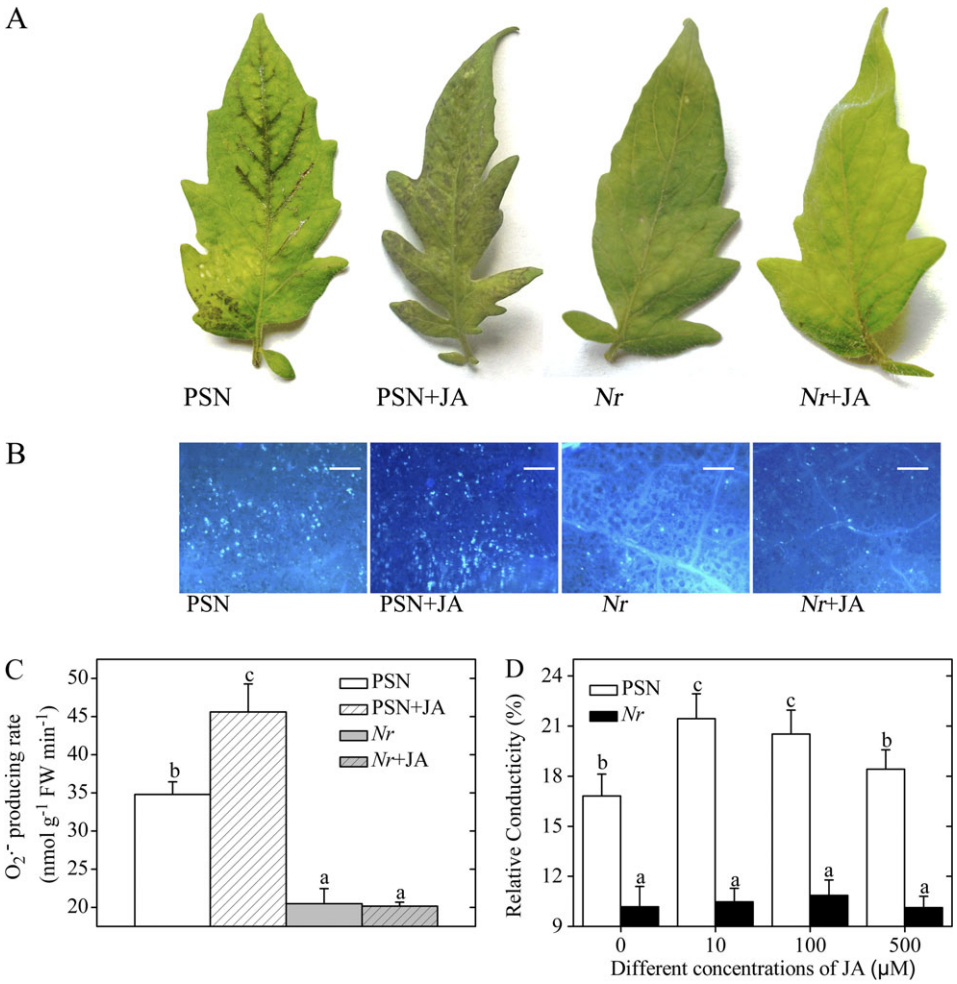

Fig. 3. Effects of exogenous JA treatment on the sensitivity of detached PSN and *Nr* leaflets to AAL toxin. Detached leaflets were incubated under continuous light at 25°C for 48 h. (A) Symptoms of PSN and *Nr* leaflets after 0.2 μM AAL toxin application with or without 100 μM JA. (B) Callose deposition in PSN and *Nr* leaves in response to AAL toxin with or without 100 μM JA. Leaflets were stained with aniline blue to detect callose. Representative microscope images are shown. Callose deposits appear as bright spots on a dark background. Scale bars represent 100 μm. (C) Quantitative measurements of the $O_2^{\cdot-}$ level in PSN and *Nr* leaflets after AAL toxin application with or without 100 μM JA. (D) Quantitative measurements of relative conductivity in leaflets of PSN and *Nr* after AAL toxin application or co-treatment with the toxin and different concentrations of JA. Error bars indicate standard deviation. For each figure, letters indicate significant differences among treatments (P <0.05, Duncan's multiple range test).

perceptional mutant *ein2* (Asai *et al.*, 2000). Transcription profiles of AAL toxin-induced cell death in the *Arabidopsis loh2* mutant plant suggested that ET-responsive genes are among the earliest to be up-regulated and AVG application reduced AAL toxin-induced PCD in *loh2* plants (Gechev *et al.*, 2004). Previous studies suggested that treatment of susceptible tomato leaflets with AAL toxin increases ACC and ET levels (Moussatos *et al.*, 1994). Inhibitors of ET biosynthesis or action markedly reduce the AAL toxin-induced lesion size and number, while the addition of exogenous ACC results in increased tissue necrosis and ET evolution (Moussatos *et al.*, 1994; Moore *et al.*, 1999). Consistent with these reports, it was found that the tomato ET-overproducing mutant *epi* evidently promoted AAL toxin-elicited PCD compared with the WT VFN8 (Fig. 2A–D). ACC application markedly promoted AAL toxin-induced cell death, while application of the inhibitor of ET action STS markedly inhibits AAL toxin-induced cell death in CA leaves (Fig. 2A–C).

Plants under abiotic and biotic stresses produce increased levels of ET, which is perceived by ET receptors and triggers cellular responses further downstream (Kim *et al.*, 2003). Six tomato ET receptors, designated LeETR1, LeETR2, LeETR3 (NR), LeETR4, LeETR5, and LeETR6, have been isolated and characterized (Klee, 2002, 2004). Each receptor gene has a distinct pattern of expression throughout development and in response to external stimuli. *NR* and *LeETR4*, but not the other genes, are induced by pathogen infection (Klee, 2002). Compared with WT PSN, the ET-insensitive mutant *Nr*, which carries a semi-dominant mutation in NR, showed markedly less necrosis and chlorosis in response to AAL toxin, suggesting

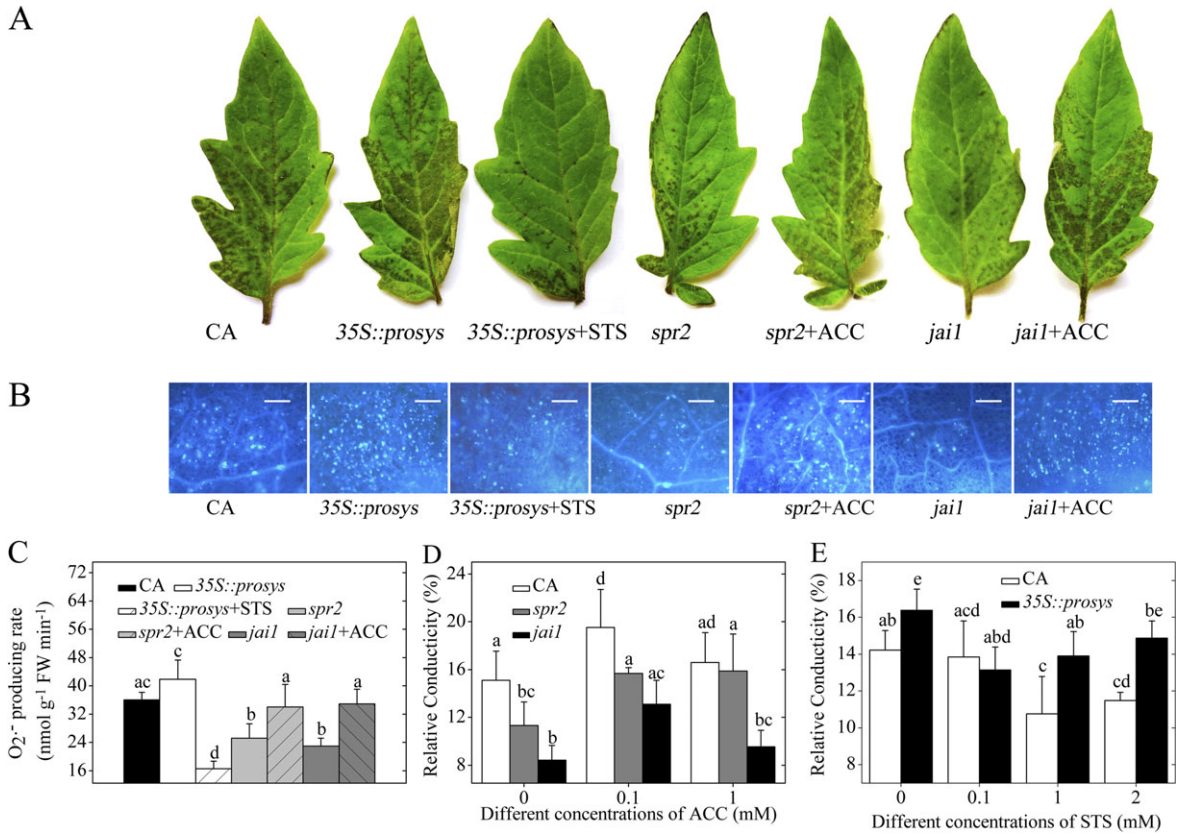

Fig. 4. Effects of exogenous ET levels on the sensitivity of detached leaflets of JA mutants to AAL toxin. (A) Symptoms of CA, *35S::prosys*, *spr2*, and *jai1* leaflets after 0.2 μM AAL toxin application or co-treatment with AAL toxin and 0.1 mM ACC or 1 mM STS. (B) Callose deposition in detached leaves of JA mutants after 0.2 μM AAL toxin application or co-treatment with AAL toxin and 0.1 mM ACC or 1 mM STS. Leaflets were stained with aniline blue to detect callose. Representative microscope images are shown. Callose deposits appear as bright spots on a dark background. Scale bars represent 100 μm. (C) Quantitative measurements of the $O_2{\cdot}^-$ level in detached leaflets of JA mutants after 0.2 μM AAL toxin application or co-treatment with AAL toxin and 0.1 mM ACC or 1 mM STS. (D) Quantitative measurements of relative conductivity in leaflets of CA, *spr2*, and *jai1* after AAL toxin application or co-treatment with the toxin and different concentrations of ACC. (D) Quantitative measurements of relative conductivity in leaflets of CA, *spr2*, and *jai1* after AAL toxin application or co-treatment with the toxin and different concentrations of ACC. (E) Quantitative measurements of relative conductivity in leaflets of CA and *35S::prosys* after AAL toxin application or co-treatment with the toxin and different concentrations of STS. Error bars indicate standard deviation. For each figure, letters indicate significant differences among treatments (P <0.05, Duncan's multiple range test).

that NR may be important in this PCD process (Moore *et al.*, 1999).

The ET pathway acts downstream of JA during the AAL toxin-induced PCD process

Since JA and ET responses are both important in cell death, it was of interest to test whether JA and ET act independently or cooperatively by addition of hormones or hormonal inhibitors to the leaves of *spr2*, *jai1*, and *Nr* mutants or *35S::prosys*, as well as their isogenic parents. The result showed that the degree of necrotic cell death and callose deposition, $O_2{\cdot}^-$ levels, and the relative conductivity in *Nr* leaves co-treated with JA and AAL toxin were similar to those of leaves treated with toxin alone (Fig. 3A–D). However, exogenous JA application markedly increased the degree of cell death and callose deposition, $O_2{\cdot}^-$ levels, and the relative conductivity in the leaves of PSN (Fig. 3A–D), consistent with those of the cv. CA.

Subsequently, *35S::prosys* leaves were co-treated with STS and AAL toxin. As shown in Fig. 4A, blocking ET perception with 1 mM STS in the presence of AAL toxin markedly reduced the amount of tissue damage in *35S::prosys* relative to the toxin treatment alone. In addition, exogenous 0.1 mM ACC application rescued AAL toxin-elicited development of disease symptoms in *spr2* and *jai1* leaflets (Fig. 4A). The callose deposition and $O_2{\cdot}^-$ production rate measured were consistent with the visible damage on *spr2*, *jai1*, and *35S::prosys* leaflets (Fig. 4B, C). Application of 0.1 mM ACC significantly increased the relative conductivity in CA and *jai1* leaves, and 0.1 mM and 1 mM ACC significantly increased the relative conductivity in *spr2* leaves (Fig. 4D). Application of 1 mM and 2 mM STS markedly decreased the relative conductivity in CA leaves, and 0.1 mM and 1 mM STS markedly decreased the

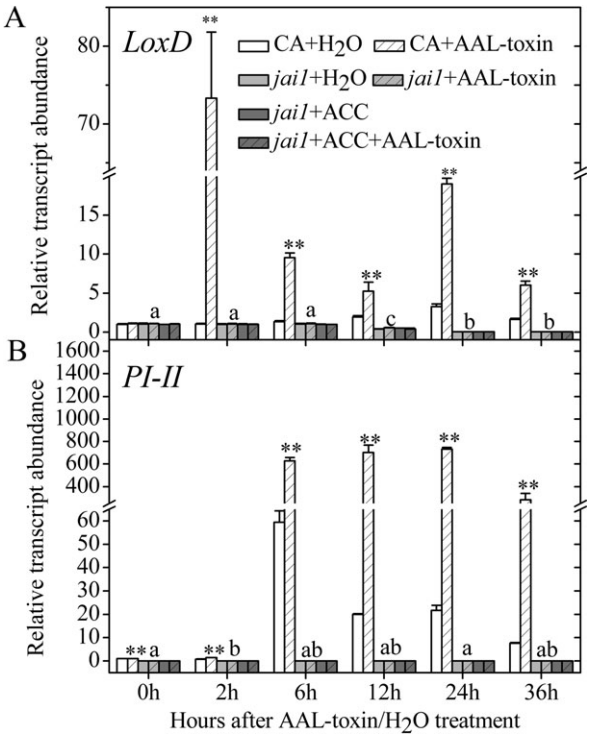

Fig. 5. Transcription patterns of the JA-biosynthetic gene *LoxD* (A) and the JA-regulated gene *PI-II* (B) in the leaflets of CA and *jai1* after AAL toxin application or co-treatment with AAL toxin and ACC. Error bars indicate standard deviation. Letters indicate significant differences in gene expression of *jai1* treated with AAL toxin alone among different time points (P <0.05, Duncan's multiple range test); asterisks indicate significant differences in gene expression of AAL toxin treatment alone between CA and *jai1* as well as in gene expression of *jai1* between treatment with AAL toxin alone and co-treatment with AAL toxin and ACC at each time point (*P <0.05; **P <0.01; Student's t-test).

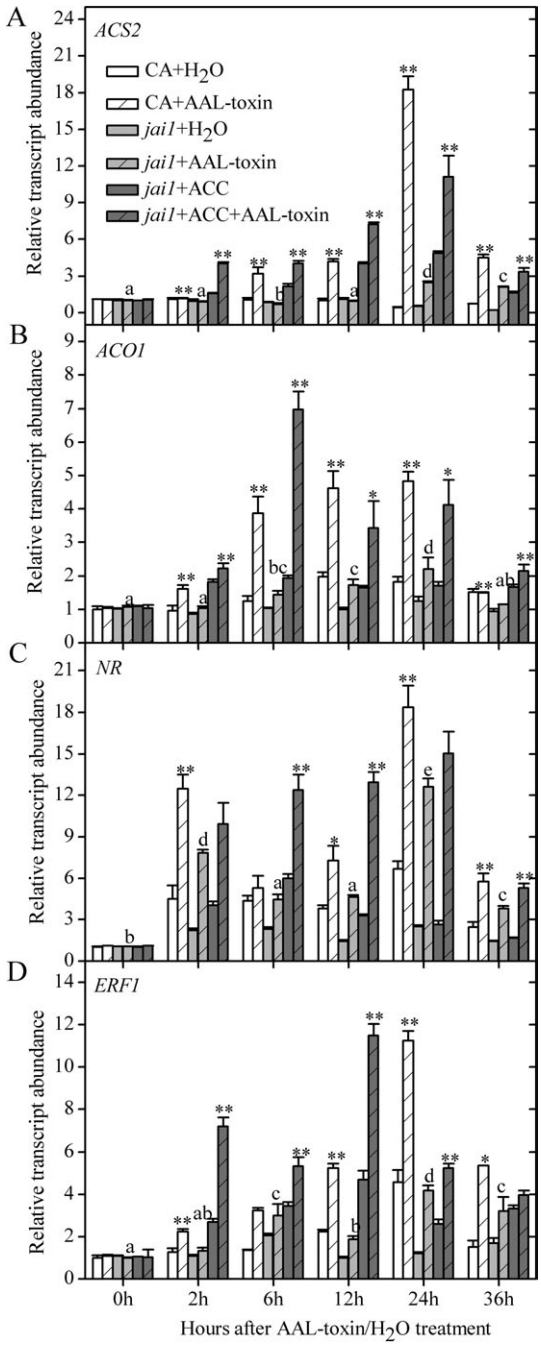

Fig. 6. Transcription patterns of ET-biosynthetic genes (*ACS2* and *ACO1*) and ET-regulated genes (*NR* and *ERF1*) in the leaflets of CA and *jai1* after AAL toxin application or co-treatment with AAL toxin and ACC. Error bars indicate standard deviation. Letters indicate significant differences in gene expression of *jai1* treated with AAL toxin alone among different time points (P <0.05, Duncan's multiple range test); asterisks indicate significant differences in gene expression of AAL toxin treatment alone between CA and *jai1* as well as in gene expression of *jai1* between treatment with AAL toxin alone and co-treatment with AAL toxin and ACC at each time point (*P <0.05; **P <0.01; Student's t-test).

relative conductivity in *35S::prosys* leaves (Fig. 4E). In addition, Fig. 4E shows that *35S::prosys* leaflets co-treated with AAL toxin and different concentrations of STS still have a significant high relative conductivity, suggesting an ET-independent pathway for AAL toxin-induced PCD. Therefore, both JA and ET pathways independently promote AAL toxin-induced PCD. Furthermore, the cell death-promoting function of ET seems to be downstream of JA signalling during the AAL toxin-induced PCD process.

AAL toxin-induced JA-related gene expression decreased in jai1 *leaves and was not restored by exogenous ACC application*

To investigate the underlying molecular mechanisms in this cell death process, the temporal expression patterns of some of the genes involved in the JA- and ET-related responses were examined by qPCR in *jai1* and *Nr* mutants, as well as their isogenic WT parents after treatment with AAL toxin with or without exogenous hormones. After *Arabidopsis loh2* mutant leaves were infiltrated with AAL toxin, expression of JA marker genes was reduced (Gechev *et al.*, 2004). Here, expression of the *LoxD* gene encoding lipoxygenase (Li *et al.*, 2004) and the *proteinase inhibitor II* (*PI-II*) gene, a marker of wounding or JA signalling (Ryan,

2000), was analysed by qPCR. As shown in Fig. 5A, the *LoxD* mRNA content in CA leaflets increased and reached a maximum (73-fold) at 2 h following toxin application, after which the transcript abundance decreased slightly, although it remained significantly higher than at 0 h. The expression of *PI-II* in CA leaflets was elevated slightly at 2 h after toxin treatment. Subsequently, it increased greatly at 6, 12, and 24 h; thereafter the expression declined slightly at 36 h (Fig. 5B). *PI-II* mRNA in the water-treated controls was also increased starting from 6 h, but to a much lesser extent than in the toxin-treated leaves (Fig. 5B). This result suggested that the accumulation of *PI-II* mRNA in the controls is more likely in response to another factor, possibly wounding. *PI-II* mRNA levels in the toxin-treated leaves were noticeably higher than those in the related water control at all time points (Fig. 5B).

The transcript levels of these two JA-related genes were greatly depressed in *jai1* leaves compared with CA leaves. In addition, when *jai1* leaves were co-treated with AAL toxin and exogenous ACC, no significant changes were observed in the transcript abundance of *LoxD* and *PI-II* compared with those treated with AAL toxin alone (Fig. 5). The above results suggested that the alleviated PCD symptoms in *jai1* leaves are associated with decreased JA signalling compared with its WT line cv. CA. Furthermore, the degree of restored PCD by exogenous ACC application in *jai1* leaves is independent of the JA pathway.

Decreased sensitivity to AAL toxin in jai1 *leaves was associated with decreased expression of ET-modulated genes*

ACC synthase (ACS) catalyses the first committed step of ET biosynthesis and is generally considered as rate limiting for ET production (Rottmann *et al.*, 1991), and ACC oxidase (ACO) catalyses the final step of ET biosynthesis (Yang and Hoffman, 1984). Accelerated ET biosynthesis in plants is frequently associated with the induction of *ACS* and *ACO* gene activation (Kim *et al.*, 2003). Moore *et al.* (1999) reported that treatment of susceptible tomato leaflets with AAL toxin increased *LeACS2* and *LeACO* mRNA levels. *ERF1* is an ET-inducible gene encoding an ERF transcription factor in tomato (Huang *et al.*, 2004). The expression of *ERF1* is induced synergistically by JA or ET in *Arabidopsis* (Lorenzo *et al.*, 2003). Berrocal-Lobo *et al.* (2004) reported that *Fusarium oxysporum*-induced *ERF1* expression depends on both functionally intact ET and JA pathways. To explore whether the decreased sensitivity to AAL toxin in *jai1* leaves is associated with the ET response, the transcript levels of ET biosynthetic genes (*ACS2* and *ACO1*) as well as ET-responsive genes (*NR* and *ERF1*) were tested in *jai1* and CA leaves following AAL toxin application. In general, AAL toxin greatly induced the expression of the four ET-related genes in cv. CA leaves. The expression of *ACS2*, *ACO1*, and *ERF1* in CA increased gradually after AAL toxin application, peaked at 24 h, and then declined at 36 h (Fig. 6A, B, D). Similarly, AAL toxin greatly induced the expression of *NR* in CA and there were

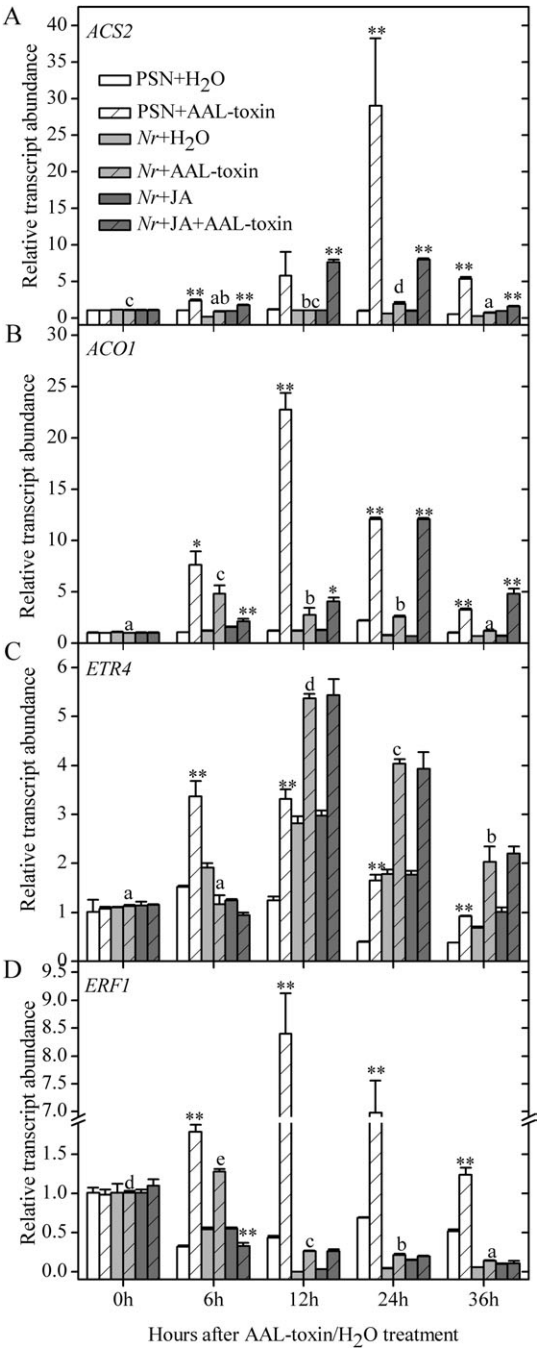

Fig. 7. Transcription patterns of ET-biosynthetic genes (*ACS2* and *ACO1*) and ET-regulated genes (*ETR4* and *ERF1*) in the leaflets of PSN and *Nr* after AAL toxin application or co-treatment with AAL toxin and JA. Error bars indicate standard deviation. Letters indicate significant differences in gene expression of *Nr* treated with AAL toxin alone among different time points ($P < 0.05$, Duncan's multiple range test); asterisks indicate significant differences in gene expression of AAL toxin treatment alone between PSN and *Nr* as well as in gene expression of *Nr* between treatment with AAL toxin alone and co-treatment with AAL toxin and JA at each time point (*$P < 0.05$; **$P < 0.01$; Student's *t*-test).

two peaks: the first at 2 h and the second at 24 h (Fig. 6C). After AAL toxin treatment, the expression of all the ET-related genes tested noticeably declined in *jai1* leaves

relative to cv. CA (Fig. 6). Furthermore, compared with AAL toxin treatment alone, co-treatment of *jai1* leaves with the toxin and exogenous ACC drastically restored the expression of *ACS2*, *ACO1*, *NR*, and *ERF1* genes. Taken together, the decreased disease symptoms in *jai1* leaves were associated with the depression of the ET pathway, and exogenous ACC restored AAL toxin-induced lesion development through the enhanced ET pathway, suggesting that the action of the JAI1-dependent JA pathway must precede ET signalling.

ET-related gene expression was decreased in Nr leaves and exogenous JA restored the expression of ET-biosynthetic genes but not the ET-responsive genes

AAL toxin induced the expression of all the ET-related genes tested, including *ACS2*, *ACO1*, *ETR4*, and *ERF1*, in cv. PSN leaves, although the trends of induction were different (Fig. 7). The expression of the four genes in PSN leaves started to increase significantly at 6 h after toxin treatment. The *ACS2* transcript peaked at 24 h and the transcript abundance of *ACO1* and *ERF1* reached a maximum at 12 h, while the *ETR4* transcript exhibited a peak at 6 h after toxin treatment and decreased gradually afterwards (Fig. 7).

Lanahan *et al.* (1994) reported similar rates of ET production by *Nr* and cv. PSN following infiltration with ACC or the bacterial pathogen *P. syringae*, suggesting that *Nr* is not impaired in any step of ET biosynthesis. ACC treatment shortened the hypocotyls of dark-grown *Nr* mutant seedlings, suggesting that the *Nr* mutant retains a residual ET response (Barry *et al.*, 2001). Here, the AAL toxin-induced expression levels of *ACS2*, *ACO1*, and *ERF1* genes in *Nr* were all markedly lower than those in cv. PSN leaves (Fig. 7A, B, D). However, *ETR4* transcript levels in *Nr* were significantly higher than in PSN leaves at 12, 24, and 36 h (Fig. 7C). This is consistent with existing findings that increased expression of the remaining receptor isoforms in receptor-deficient lines tend to compensate partially for the missing receptors at the level of mRNA expression (Tieman *et al.*, 2000). Furthermore, the reduced relative conductivity triggered by AAL toxin in *Nr* (Fig. 3D) implied that the altered composition of receptor isoforms (NR and LeETR4) did not completely compensate the output of ET signalling, which was consistent with the findings reported by Tieman *et al.* (2000).

Compared with toxin treatment alone, co-treatment of *Nr* leaves with AAL toxin and exogenous JA restored the *ACS2* and *ACO1* mRNA contents (Fig. 7A, B). However, the toxin-induced transcript abundance of ET-responsive genes *ETR4* and *ERF1* in *Nr* leaves was not significantly influenced by exogenous JA application (Fig. 7C, D). Therefore, it is concluded that the JA pathway must act upstream of ET biosynthesis in the AAL toxin-induced PCD process.

Conclusions

In this study, a pathogen-free model system was employed to investigate the function of JA and ET signalling as well as the interactions between the two hormones in regulating the AAL toxin-induced PCD process. Both JA and ET promote AAL toxin-induced cell death alone in detached tomato leaves. In addition, JAI1 receptor-dependent JA signalling promotes PCD through ET pathway action, at some point upstream of ET biosynthesis (Fig. 8). The expression of some JA- and ET-related genes was detected, but the possibility that other genes may also participate in this process could not be excluded. Interaction and balance between the hormone pathways may serve to fine-tune disease propagation or containment processes, resulting in different lesion sizes and formation dynamics.

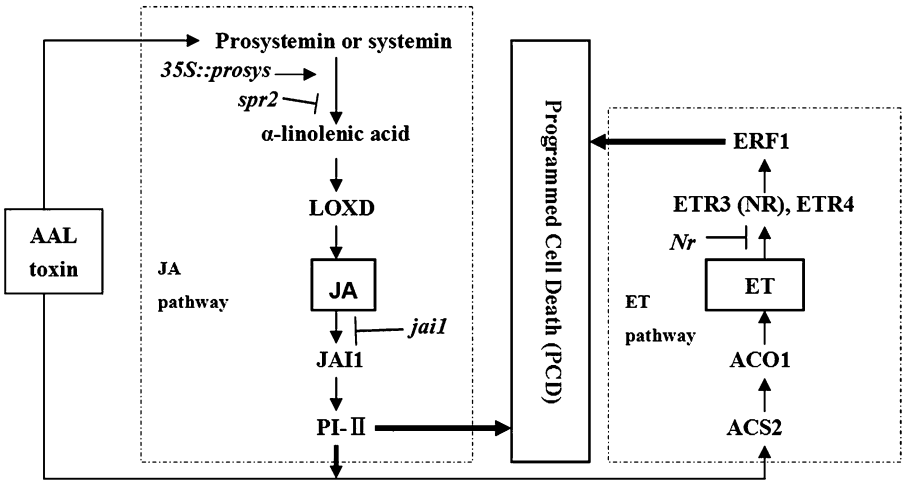

Fig. 8. A proposed model of synergistic interactions between ET and JA signalling pathways in the regulation of AAL toxin-induced PCD in detached tomato leaves. Both JA and ET pathways promote AAL toxin-induced PCD alone. Furthermore, JA signalling mediated through JAll seems to act upstream of ET biosynthesis in this PCD process. Simplified JA and ET pathways are included in two rectangles composed of dashed lines and dots, respectively. Mutant lines in which the components of ET or JA signalling alter were used in this study. Solid arrows represent positive interactions, whereas T-bars indicate inhibition.

The findings have extended our understanding of JA and ET interaction during the AAL toxin-induced PCD process, which differs from what is known in *Arabidopsis*. JA signalling promotes AAL toxin-induced tomato cell death, while JA marker genes are either not induced or down-regulated in *Arabidopsis* leaves after AAL toxin application (Gechev *et al.*, 2004), which is consistent with the existing reports that the hormones led to increased or decreased disease severity depending on the plant–pathogen combination. The results are helpful to future effort in controlling plant cell death through the manipulation of disease-related signalling molecules.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Effects of MeJA on the sensitivity of tomato leaf discs to AAL toxin.

Figure S2. The plants of *spr2*, *def1*, *35S::prosys*, and *jai1*, as well as their parental line cultivar Castlemart (CA) used in this study.

Figure S3. Treatment method of detached tomato leaflets.

Figure S4. The relative conductivity in leaflets of cv. CA, cv. PSN, *spr2*, *jai1*, *35S::prosys*, and *Nr* was measured after exogenous JA, STS, or ACC treatment alone for 48 h.

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