

Defence responses regulated by jasmonate and delayed senescence caused by ethylene receptor mutation contribute to the tolerance of petunia to *Botrytis cinerea*

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SUMMARY

Ethylene and jasmonate (JA) have powerful effects when plants are challenged by pathogens. The inducible promoter-regulated expression of the *Arabidopsis* ethylene receptor mutant *ethylene-insensitive1-1* (*etr1-1*) causes ethylene insensitivity in petunia. To investigate the molecular mechanisms involved in transgenic petunia responses to *Botrytis cinerea* related to the ethylene and JA pathways, *etr1-1*-expressing petunia plants were inoculated with *Botrytis cinerea*. The induced expression of *etr1-1* by a chemical inducer dexamethasone resulted in retarded senescence and reduced disease symptoms on detached leaves and flowers or intact plants. The extent of decreased disease symptoms correlated positively with *etr1-1* expression. The JA pathway, independent of the ethylene pathway, activated petunia ethylene response factor (*PhERF*) expression and consequent defence-related gene expression. These results demonstrate that ethylene induced by biotic stress influences senescence, and that JA in combination with delayed senescence by *etr1-1* expression alters tolerance to pathogens.

INTRODUCTION

The death of cells can be a programmed event that occurs when plants are attacked by pathogens (Greenberg, 1997). *Botrytis cinerea*, a model necrotrophic pathogen, triggers the host cell death response because it produces toxins. A hypersensitive reaction (HR) occurs at the site of contact. In *Arabidopsis*, the HR facilitates further infection with *B. cinerea* (Govrin and Levine, 2000). For the full pathogenicity of *B. cinerea* in tobacco, an HR is required (Dickman *et al.*, 2001). Furthermore, delayed or reduced cell death *Arabidopsis* mutants are more resistant than wild-type plants to *B. cinerea* (van Baarlen *et al.*, 2007).

Ethylene, a gaseous hormone, regulates different processes during the lifetime of the plant. Ethylene is involved in the regulation of programmed cell death (PCD) during plant–pathogen interactions (Greenberg, 1997). Ethylene participates in cell death processes (Lam *et al.*, 1999), as shown in the *Arabidopsis* lesion mimic mutant *vad1-1* affecting *ethylene-insensitive4* (*EIN4*) (Bouchez *et al.*, 2007). Ethylene perception and signalling transduction have significant roles in mediating the effects of ethylene during senescence. However, the five *Arabidopsis* ethylene receptors have different roles during plant responses to the toxin fumonisin B1, and the mechanisms of each pathway may differ (Plett *et al.*, 2009).

Ethylene also participates in plant defence responses to pathogens. Ethylene perception and signalling mutants have different disease symptoms when challenged by pathogens; some responses lead to resistance or susceptibility (Knoester *et al.*, 1998), but the response can also be the same as in wild-type plants (Hoffman *et al.*, 1999; Thomma *et al.*, 1999). For example, the ethylene-insensitive tomato mutant *Never ripe* (*Nr*) has the same visible symptoms as wild-type plants infected by *B. cinerea* (Diza *et al.*, 2002), but is more resistant to *Fusarium oxysporum* (Lund *et al.*, 1998). However, detached *Nr* leaves have also been reported to be more susceptible to *B. cinerea* (Audenaer *et al.*, 1999). When infected by the bacterial pathogen *Clavibacter michiganensis* ssp. *michiganensis*, which causes plant wilt and canker symptoms, *Nr* shows delayed symptoms (Balaji *et al.*, 2008). Regardless of the differences in plant responses to a pathogen, exposure to and perception of ethylene are known to activate plant defence-related proteins, such as the pathogenesis-related (PR) proteins (van Kan *et al.*, 1995) *PR1b* and *PR5* (*osmotin*) (Xu *et al.*, 1994), *PR3* (Lorrain *et al.*, 2004) and *plant defensin1.2* (*PDF1.2*). These proteins are also known to be activated by the jasmonate (JA) pathway of plant responses (Kunkel and Brooks, 2002; Penninckx *et al.*, 1998). JAs are involved in the mediation of plant responses to pathogens (Farmer and Ryan, 1992; Glazebrook, 2005), and can act synergistically with (Penninckx *et al.*, 1998; Thomma *et al.*, 1999; Xu *et al.*, 1994) or independently of

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(Díaz *et al.*, 2002) ethylene in different plants, especially when attacked by necrotrophic pathogens (Glazebrook, 2005).

Ethylene-insensitive1-1 (etr1-1)-expressing plants are insensitive to ethylene (Bleecker *et al.*, 1988; Hall *et al.*, 1999; Hua *et al.*, 1998). Here, we used a reproducible and comprehensive *B. cinerea* infection system on petunia plants in which *etr1-1* was inducibly expressed. We investigated the disease symptoms of intact plants, detached flowers and leaves from two transgenic lines with different levels of *etr1-1* expression. The analysis measured the proportion of expanding lesions, the lesion growth rate (Díaz *et al.*, 2002), disease severity and disease incidence. We also compared the effects of *B. cinerea* infection on the PCD response in these lines.

Our objectives were to determine the role of induced *etr1-1* expression on senescence induced by biotic stress, i.e. *B. cinerea* infection, and the roles of ethylene and JA during petunia responses to *B. cinerea*.

RESULTS

Fungal growth and disease progress

For coordinated and reproducible *B. cinerea* infection of petunia plants, we inoculated the detached leaves and flowers and intact plants of two lines, E7H and E9G, in which *etr1-1* expression was induced with dexamethasone (DEX). The infection of detached petunia leaves was essentially the same as the infection of tomato leaves as described by Benito *et al.* (1998) and Díaz *et al.* (2002). Primary lesions formed, followed by a quiescent period, and subsequently by expanding lesions. As shown in Fig. 1a, the primary necrotic lesion formed at 1–2 days post-inoculation (dpi). Primary lesions were observed at 80% of the infected sites of leaves treated with dimethylsulphoxide (DMSO); primary lesions formed on 20% of the sites of leaves treated with DEX. The expansion of lesions on DEX-induced leaves was slower than on DMSO-treated

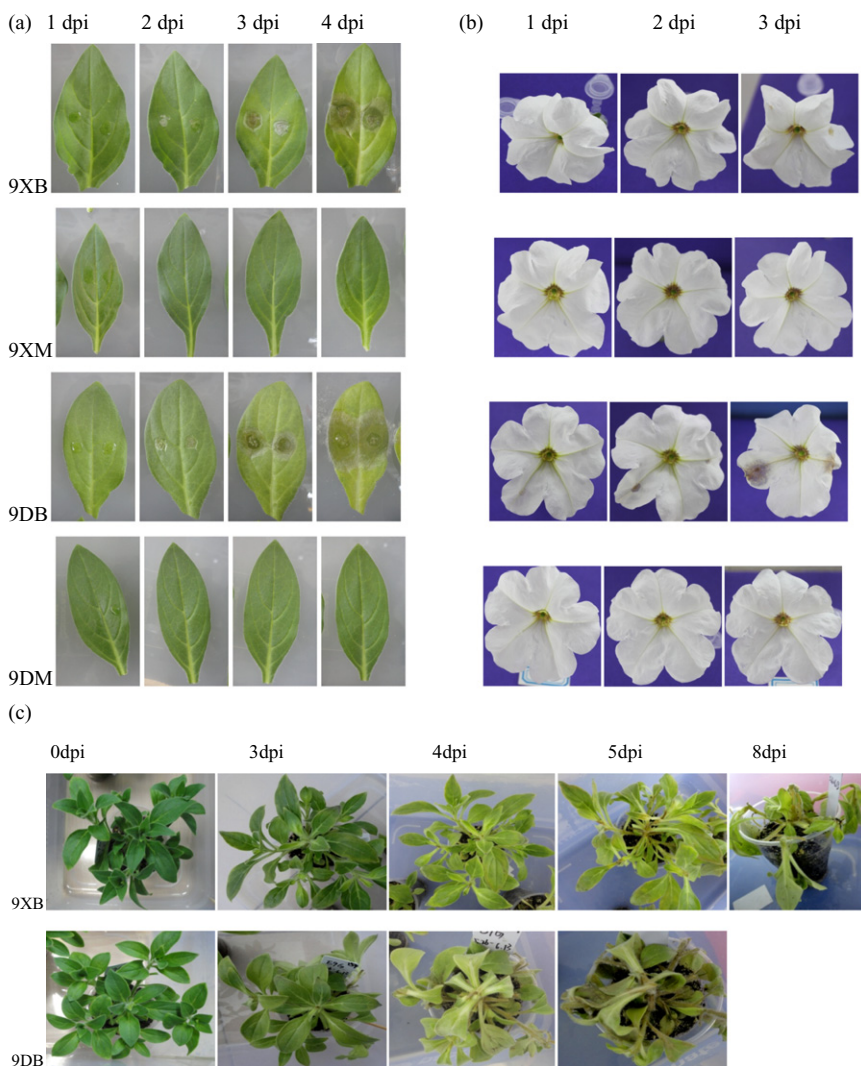


Fig. 1 Representative phenotypes of dexamethasone (DEX) and dimethylsulphoxide (DMSO) treatment on E9G inoculated with *Botrytis cinerea*. (a) Disease symptoms on the fourth leaves from the top *in vitro*. (b) Disease phenotype of fully opened flowers before anther dehiscence *in vitro*. (c) Disease phenotype of 8-week-old plantlets. 9XB, DEX treatment–*B. cinerea* inoculation on E9G; 9DB, DMSO treatment–*B. cinerea* inoculation on E9G; 9XM, DEX treatment–mock inoculation on E9G; 9DM, DMSO treatment–mock inoculation on E9G. dpi, days post-inoculation.

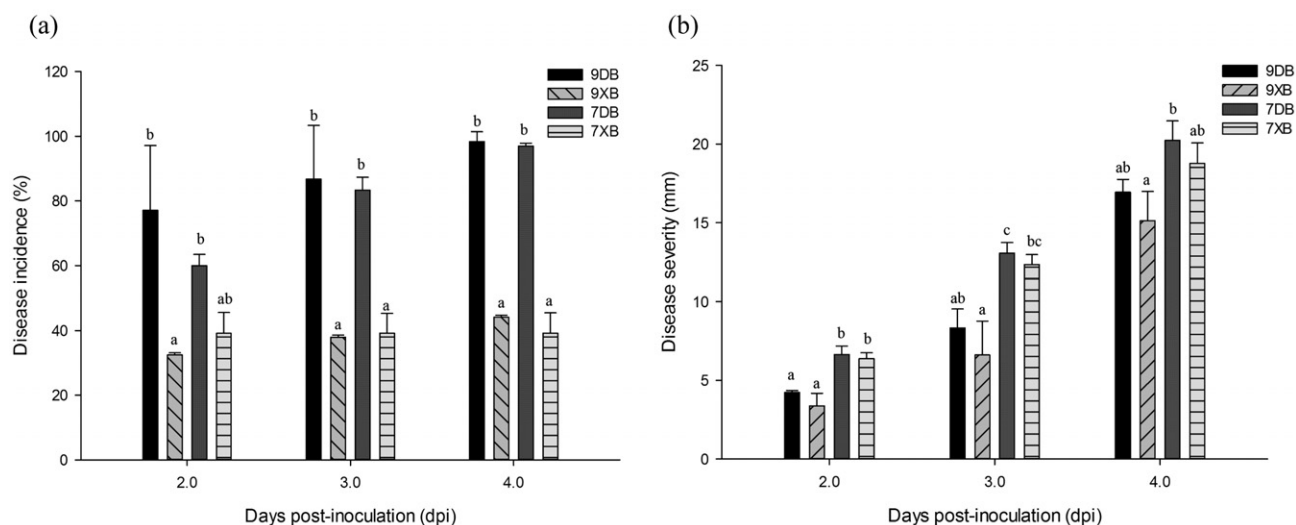


Fig. 2 Susceptibility of E9G and E7H to *Botrytis cinerea* with dexamethasone (DEX) or dimethylsulphoxide (DMSO) treatment. (a) Disease incidence (percentage of inoculation sites with expanding lesions) of the fourth leaves from the top on E7H and E9G lines *in vitro*. (b) Disease severity (diameter of expanding lesions) of the fourth leaves from the top on E7H and E9G lines *in vitro*. Mean values are shown from three independent bioreplicates [error bars, \pm standard error (SE)] containing at least 40 leaves (80 droplets) for every experiment. Different letters indicate significant differences between treatments of the two lines at a given time point ($P \leq 0.05$). 9XB, DEX treatment–*B. cinerea* inoculation on E9G; 9DB, DMSO treatment–*B. cinerea* inoculation on E9G; 7XB, DEX treatment–*B. cinerea* inoculation on E7H; 7DB, DMSO treatment–*B. cinerea* inoculation on E7H.

Table 1 Susceptibility of E7H and E9G plantlets with dexamethasone (DEX) or dimethylsulphoxide (DMSO) treatment to *Botrytis cinerea*. Data were pooled from two independent experiments. Each plant was considered as a replicate. Different letters indicate significant differences between the treatment of two lines at a given time point ($P \leq 0.05$, \pm SD, $n = 10$).

Treatment	Healthy plants* (%)		Expanding lesion per plant* (%)		Lesion expansion rate† (mm/day)	
	E7H	E9G	E7H	E9G	E7H	E9G
DMSO-B	0	0	6.40 \pm 2.01b	3.50 \pm 2.4a	5.23 \pm 1.23b	3.91 \pm 2.23b
DEX-B	28.33	46.77	3.90 \pm 0.99a	2.30 \pm 1.49a	4.04 \pm 1.47a	1.87 \pm 1.05a
DMSO-M	100	100	0	0	0	0
DEX-M	100	100	0	0	0	0

B, *Botrytis*; M, mock; SD, standard deviation.

*At 5 days post-inoculation (dpi).

†Calculated over the period from 5 to 6 dpi.

leaves at 2 dpi (Fig. 1a). The disease incidence in leaves treated with DMSO was approximately 100%, whereas more than 50% of DEX-treated leaves still appeared healthy (Fig. 2a). The disease severity of DEX-treated leaves from the E9G and E7H (Fig. S1) lines was reduced at 3–4 dpi, although not significantly, relative to that of DMSO-treated leaves (Fig. 2b).

To examine other tissues for their responses to *B. cinerea*, we also inoculated detached flowers (Fig. 1b) and intact plantlets (Fig. 1c). Similar to the disease symptoms on detached leaves, the proportion of expanding lesions and the lesion growth rate were reduced in DEX-treated plantlets of the two lines relative to the DMSO-treated control (Table 1). At 5 dpi, 28.33% and 46.77% of the E7H and E9G plantlets, respectively, were healthy. At 8 dpi, the infection was completely subdued in plantlets treated with DEX, whereas, in control plants, saturating infections occurred at 5 dpi (Fig. 1c). The delayed disease progress was also reflected by a statistically significantly decreased number of expanding lesions per flower, as well as a reduced growth rate of the expanding

lesions on petunia flowers treated with DEX, when compared with DMSO-treated flowers (Table 2).

We analysed the expression of *etr1-1* produced by DEX induction by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. 3a). As expected, the expression level of *etr1-1* increased in the leaves of DEX-treated intact plants after mock infection as well as after *B. cinerea* inoculation. The expression level of *etr1-1* reached a maximum at 3 days and decreased at 4 days after mock infection. However, the expression only increased slightly in *B. cinerea*-infected plants induced with DEX.

To determine whether the decrease in disease severity and expanding lesions was consistent with a decrease in fungal biomass, gene expression of *B. cinerea actin* (*Bcactin*), a marker for actively growing *B. cinerea* (Benito *et al.*, 1998; Díaz *et al.*, 2002), was assessed (Fig. 3b). Disease severity-associated gene expression was found (Figs 1c and 3b). A 25-fold increase in *Bcactin* expression was observed in DMSO-treated infected tissue at 4–5 dpi compared with control plants treated with DEX, a result

Treatment	Healthy flowers* (%)		Expanding lesion per flower* (%)		Lesion expansion rate† (mm/day)	
	E7H	E9G	E7H	E9G	E7H	E9G
DMSO-B	0	0	100 ± 0.00c	100 ± 0.00c	4.58 ± 1.33d	2.68 ± 0.82c
DEX-B	65	60	75 ± 0.89b	60 ± 0.67a	2.26 ± 0.63b	1.43 ± 0.22a
DMSO-M	100	100	0	0	0	0
DEX-M	100	100	0	0	0	0

B, *Botrytis*; M, mock; SE, standard error.

*At 5 days post-inoculation (dpi).

†Calculated over the period from 5 to 6 dpi.

Table 2 Susceptibility of E7H and E9G flowers with dexamethasone (DEX) or dimethylsulphoxide (DMSO) treatment to *Botrytis cinerea*. Data were pooled from two independent experiments, with eight flowers per treatment. Different letters indicate significant differences between the treatment of two lines at a given time point ($P \leq 0.05$, \pm SE, $n = 8$).

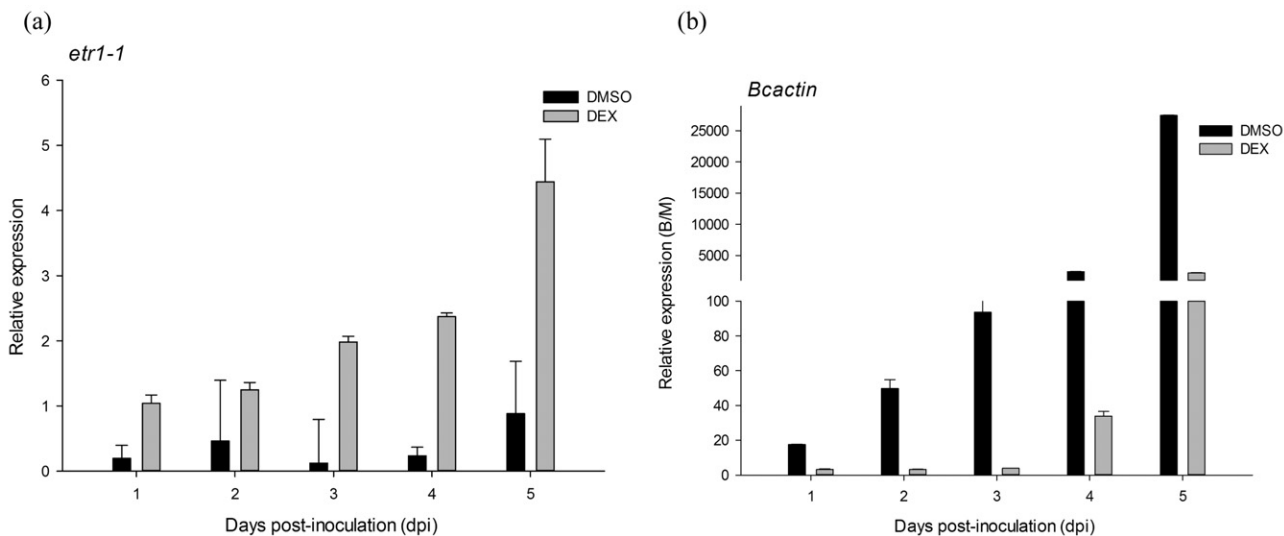


Fig. 3 Gene expression patterns in E9G plantlets infected with *Botrytis cinerea* with dexamethasone (DEX) or dimethylsulphoxide (DMSO) treatment.

Eight-week-old E9G plantlets, which were irrigated for 4 days with 30 μ M DEX or 0.18% DMSO (v/v), were sprayed with 300 μ L of a suspension of *B. cinerea* with 10 mM glucose and 6.7 mM potassium phosphate (pH 6.0) or mock sterile H₂O containing the incubation buffer. Total RNA was extracted from leaf samples collected at every day post-inoculation (dpi). cDNA analysis was performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) amplification with specific primers designed by PRIMER 3 (Table S1). Transcript levels were normalized to 26S rRNA. Data represent two independent replicates [\pm standard error (SE), $n = 10$]. B/M, *Botrytis/mock*.

consistent with the visually accelerated and increased disease symptoms (Fig. 1c and Table 1).

Progressive senescence in infected GVG::etr1-1 transgenic petunias

Botrytis cinerea kills host cells by producing phytotoxins, such as botrydial, which induces chlorosis and cell collapse, thereby facilitating penetration and colonization (Choquer *et al.*, 2007). This necrotrophic pathogen thrives on dead cells (Plett *et al.*, 2009). Chlorophyll loss is known to be an indicator of senescence (Mach *et al.*, 2001; Plett *et al.*, 2009) and has been used to quantify differences in cell death (Pegadaraju *et al.*, 2005). To determine the degree of cell death induced by *B. cinerea* and the role of ethylene in cell death, the leaves of E7H and E9G plants treated with DEX or DMSO were investigated for chlorophyll loss. As shown in Fig. 4, infected leaves lost chlorophyll. The chlorophyll loss rate in DEX-induced tissues (7.77% and 14.27% on intact and

detached leaves, respectively) was less than half than (14.92% and 28.21%) the DMSO treatment (Fig. 4a, b).

Another means by which *B. cinerea* may cause host cell death is through the oxidative burst and the release of reactive oxygen species (ROS); these events occur during penetration (Tenberge *et al.*, 2002; Tenberge, 2004) and primary lesion formation (Choquer *et al.*, 2007; van Kan, 2006). The HR, a type of PCD thought to promote *B. cinerea* growth, is required for the rapid production of ROS (van Kan, 2006). We measured malondialdehyde (MDA) (Fig. 4c), an aldehyde product of lipid peroxidation, considered to be a reliable marker of plant senescence, and the enzymatic antioxidants catalase (CAT) (Fig. 4d) and superoxide dismutase (SOD) (Fig. 4e), which help to mitigate the toxic effects of active oxygen species; the expression of these markers changed in infected and treated tissues. Detached leaves were selected to measure MDA and antioxidant enzymes, because the three colonization phases of *B. cinerea* were easier to recognize in this tissue. A significant increase in the MDA content of infected leaves

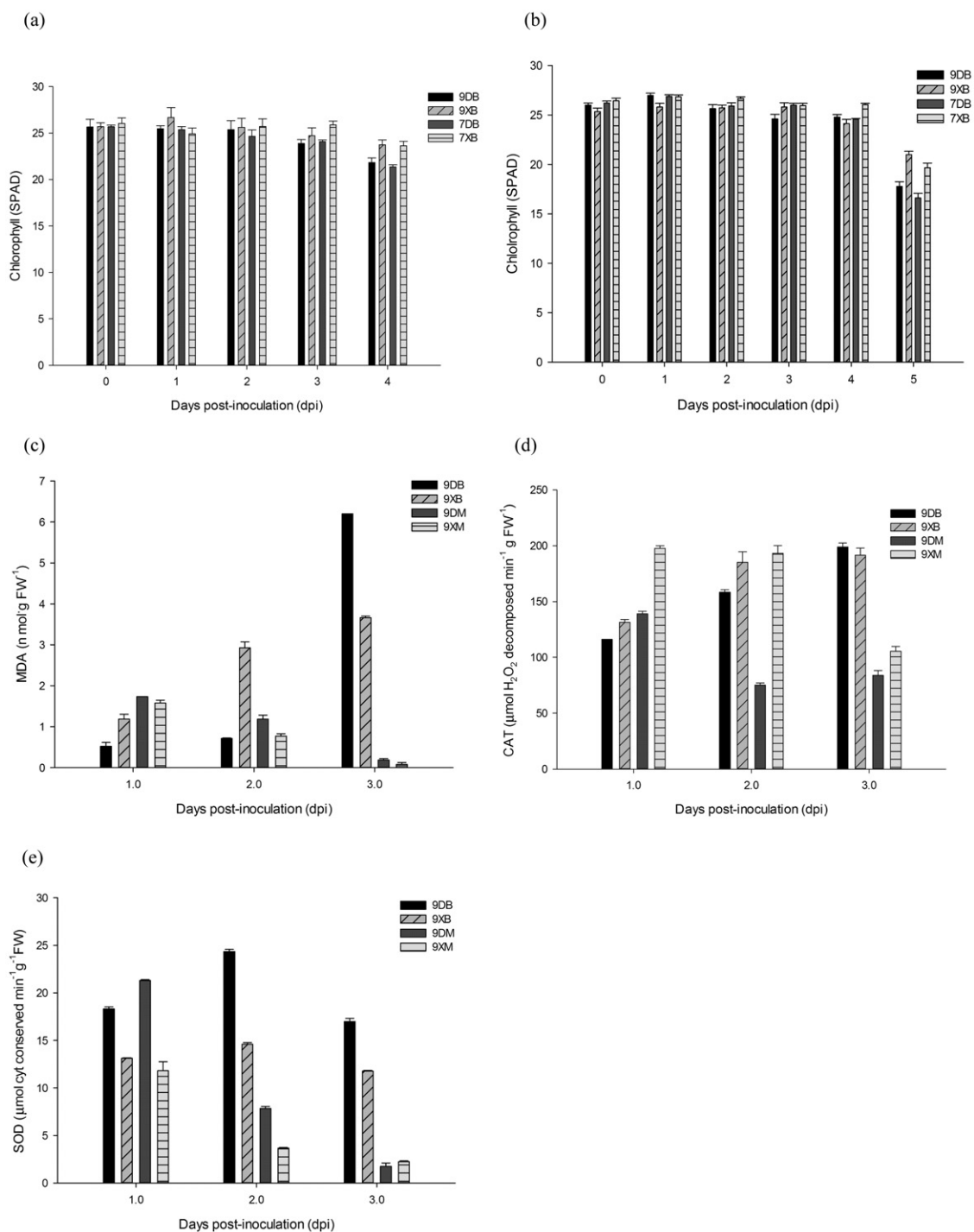


Fig. 4 Physiological markers of senescence in petunias. The fourth leaf from the top of 8-week-old plantlets was collected and painted with 30 μM dexamethasone (DEX) or 0.18% dimethylsulphoxide (DMSO) (*v/v*) in 0.6% agar medium. After 2 days, leaves were inoculated with two 10- μL droplets using a spore suspension of *Botrytis cinerea* or mock solution. (a) The chlorophyll content in E9G plantlets infected with *B. cinerea*. Data represent two independent replicates (\pm standard error (SE), $n = 10$). The chlorophyll content of detached leaves (b), malondialdehyde (MDA) level of detached leaves (c), catalase (CAT) level of detached leaves (d) and superoxide dismutase (SOD) level of detached leaves (e) at the indicated time points *in vitro*. Data represent three independent experiments (\pm SE, $n = 120$). Data were subjected to one-way analysis of variance (ANOVA). Significant differences are indicated by letters ($P < 0.05$). 9XB, DEX treatment-*B. cinerea* inoculation on E9G; 9DB, DMSO treatment-*B. cinerea* inoculation on E9G; 7XB, DEX treatment-*B. cinerea* inoculation on E7H; 7DB, DMSO treatment-*B. cinerea* inoculation on E7H; 9XM, DEX treatment-mock inoculation on E9G; 9DM, DMSO treatment-mock inoculation on E9G; FW, fresh weight.

was found, regardless of DEX treatment (Fig. 4c), and an increase in MDA content was not observed in mock-treated leaves; therefore, this response was *B. cinerea* dependent. The greatest increase in the MDA concentration was observed in leaves that were more severely infected. CAT activity was increased significantly in infected leaves; however, SOD activity increased at 2 dpi, and then declined at 3 dpi (Fig. 4e). In contrast with these results in infected leaves, MDA and CAT levels were reduced significantly in mock-treated leaves. This demonstrates that detached mock-treated leaves are not in a stage of senescence, consistent with the visible phenotype (Fig. 1a) and chlorophyll content (Fig. 4b).

In order to determine whether *B. cinerea* induces senescence in GVG::*etr1-1* transgenic petunias after treatment with DEX or DMSO, as in *Arabidopsis* and tomato plants (Swartzberg *et al.*, 2008), and whether ethylene regulates the progression of senescence induced by biotic stress, we monitored the expression of nine petunia *cysteine protease* (*PhCP*) genes. Increased expression of CPs has been detected during petunia flower senescence (Jones *et al.*, 2005) and *PhCPs* have been reported to be regulated during senescence by different types of stress (Jones *et al.*, 2005). As shown in Fig. 5, the transcriptional accumulation of the tested *PhCPs* was activated by *B. cinerea* infection, with the exception of *PhCP2* and *PhCP4*, which displayed much lower expression levels during the entire monitoring period. The expression level of *PhCP8* was highest. The peak of expression of most *PhCPs* was at 2 dpi, except for *PhCP9*, which peaked at 4 dpi.

JA action on infected GVG::*etr1-1* transgenic petunias

To acquire information on ethylene activity during plant–pathogen interactions, we monitored the expression of the genes involved in the ethylene biosynthesis and signalling pathways. 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO) converts 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Kende, 1993), is a critical enzyme in the ethylene biosynthesis pathway (Yang and Hoffman, 1984) and is an appropriate indicator of ethylene production (Balague *et al.*, 1993; Barry *et al.*, 1996; Hamilton *et al.*, 1990; Holdsworth *et al.*, 1987; Liu *et al.*, 1985). As shown in Fig. 6, the *PhACO* gene expression level was increased by *B. cinerea* in infected leaves, regardless of DEX treatment. The expression levels of four genes of the ethylene pathway, *ETR2* (*ethylene receptor 2*), *ERS1* (*ethylene response sensor 1*), *EIN2* (*ethylene-insensitive 2*) and *EIL1* (*EIN3-like1*), were repressed during the entire inoculation period, apart from at 1 dpi when maximum transcript abundance was observed on DEX-treated leaves. However, in *B. cinerea*-inoculated DMSO-treated plants, the abundance of transcripts of *ETR2*, *ERS1*, *EIN2* and *EIL1* was increased and reached a maximum at 3 or 4 dpi.

To gain insight into whether the JA pathway was activated when the ethylene pathway was blocked during this plant–pathogen interaction, we compared the expression of *allene oxide*

cyclase (*AOC*) (Ahkami *et al.*, 2009) and *CORONATINE INSENSITIVE 1* (*COI1*) (Breuillin *et al.*, 2010). *AOC* and *COI1* are important for JA biosynthesis and signalling transduction (Feys *et al.*, 1994; Wasternack and Hause, 2002; Xie *et al.*, 1998). The expression of *AOC* and *COI1* was activated by *B. cinerea*, reaching a peak at 1 and 3 dpi in DEX-treated leaves (Fig. 6). The expression of *PhCOI1* was inhibited, although *PhAOC* expression was increased, in DMSO-treated infected leaves (Fig. 6).

ERF expression profiles in infected GVG::*etr1-1* transgenic petunias

The expression of ethylene response factors (ERFs), downstream components of the ethylene signalling pathway, was analysed (Fig. 7). *ERF* transcripts accumulated when tissues were inoculated with *B. cinerea*, regardless of DEX or DMSO treatment, and reached peaks at various time points post-inoculation. The maximum expression of certain *ERF* genes, such as *ERF2*, *ERF3*, *ERF4* and *ERF7*, occurred at 3 dpi in DEX-treated leaves; however, *ERF8*, *ERF4*, *ERF6*, *ERF8* and *ERF13* were maximal at 4 dpi in DMSO-treated tissues.

Defence-related gene expression profile in infected GVG::*etr1-1* transgenic petunias

RT-PCR amplification was used to evaluate whether the expression of defence-related genes was increased following infection with *B. cinerea* on DEX- and DMSO-treated tissues (Fig. 8). As shown in Fig. 8, the expression of genes encoding for petunia defence-related proteins was activated after *B. cinerea* inoculation of plants with or without DEX induction. The maximum transcript accumulation of *PR6*, *OSM* (*osmotin*; *PR5*) and *Prxs1* (*peroxidase1*) was higher in DEX-treated leaves than in DMSO-treated leaves; by contrast, the expression levels of *EX-CHI* (*extracellular chitinase*) and *AC-CHI* (*acidic chitinase*) were lower in DEX-treated leaves than DMSO-treated leaves. The maximum expression level of all tested defence-related genes, with the exception of *D1* (*defence1*), was seen at 3 dpi on DEX-induced samples (Fig. 8a). However, the peak of gene transcript accumulation of the tested defence-related proteins occurred at 4 dpi, except for *Prxs1* (at 1 dpi), in DMSO-treated plants (Fig. 8b).

DISCUSSION

The extent of delayed disease symptoms is related to the level of *etr1-1* induction

The ethylene pathway, including ethylene biosynthesis and perception, is known to be involved in the response of plants to abiotic and biotic stresses (Abeles *et al.*, 1992; Reid, 1995). Previous research has demonstrated that ethylene-insensitive

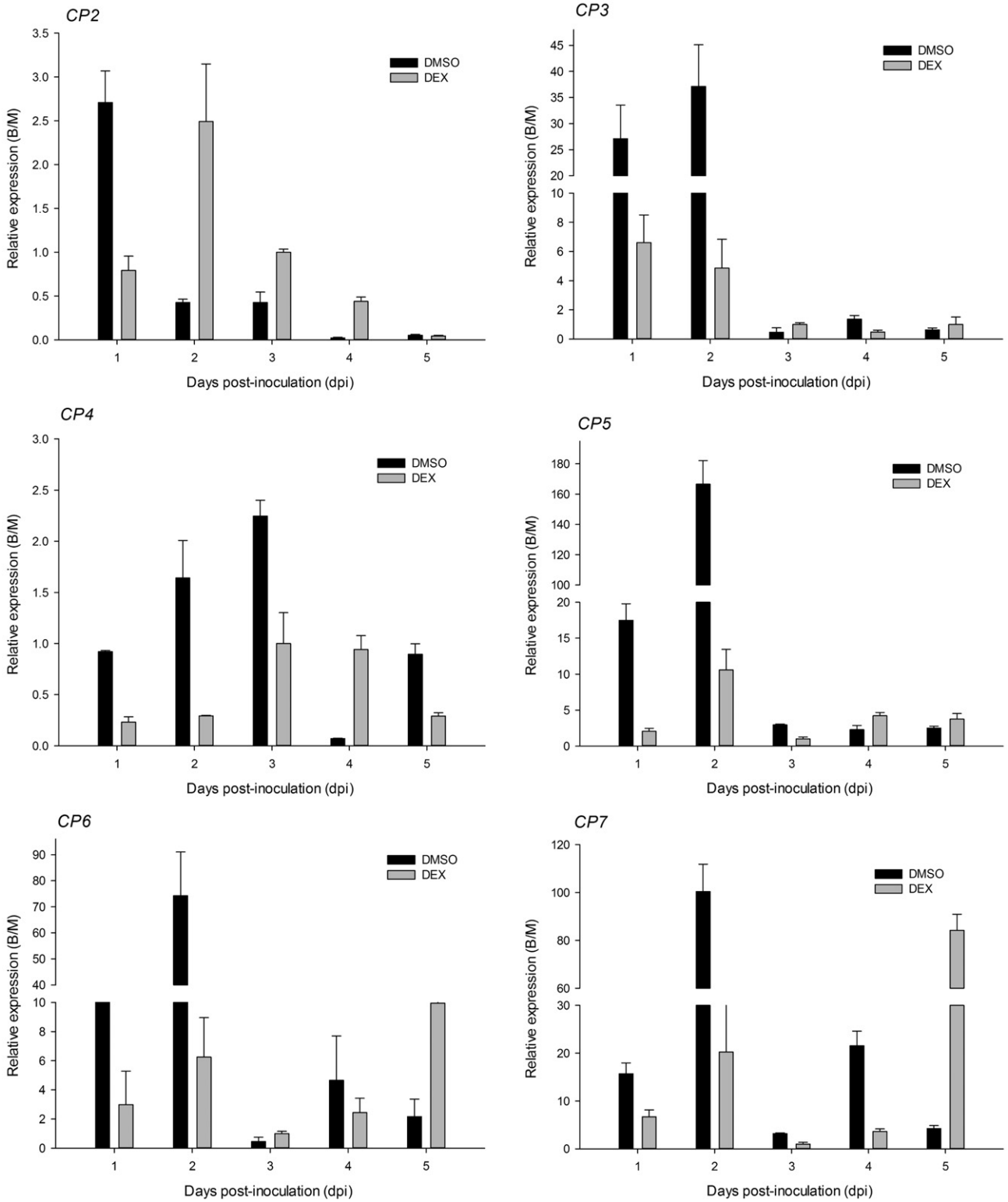


Fig. 5 Gene expression patterns of nine cysteine proteases (CPs) in E9G plantlets infected with *Botrytis cinerea*. B/M, *Botrytis*/mock.

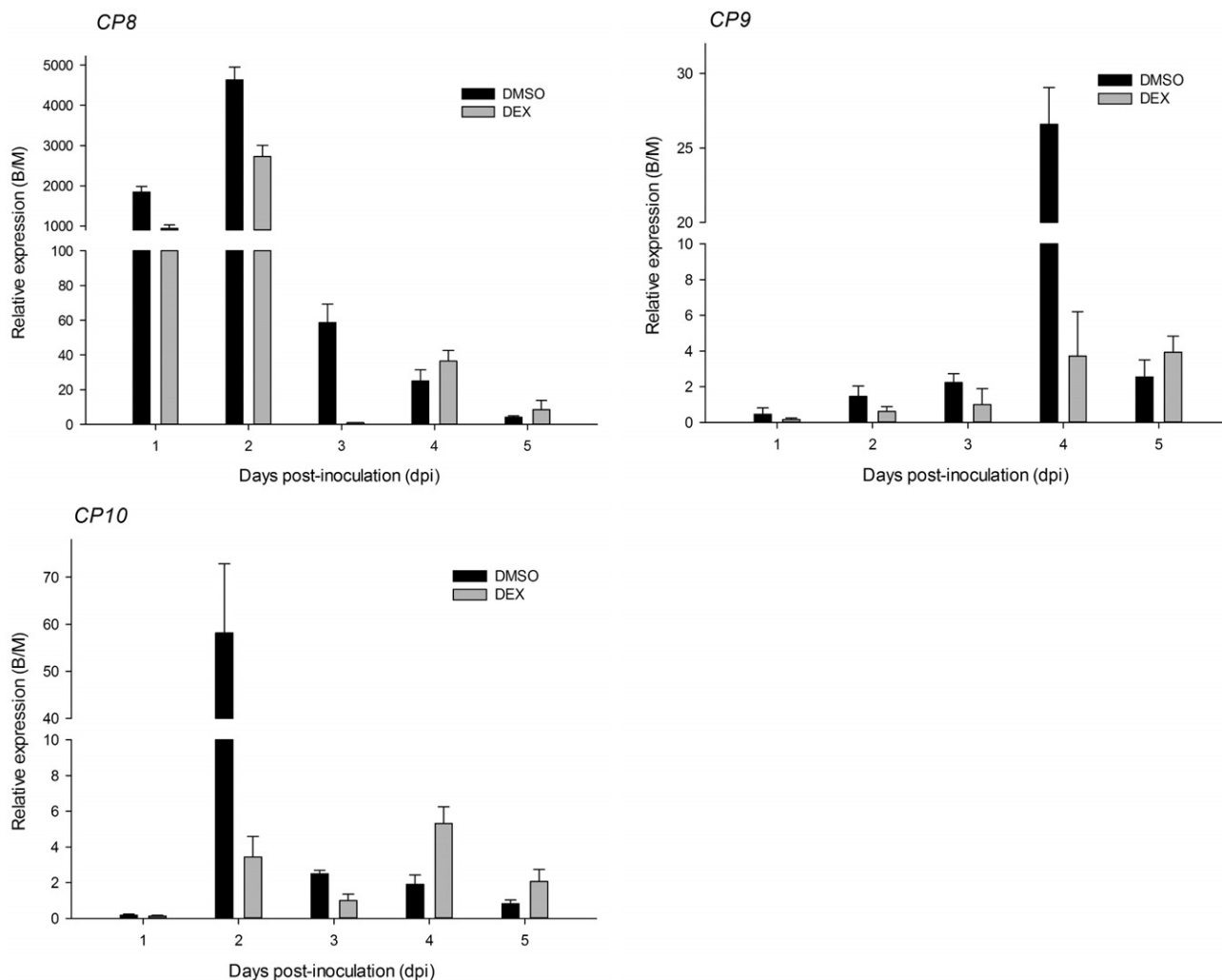


Fig. 5 Continued

tobacco displays disease symptoms when infected with soil-borne fungi (Knoester *et al.*, 1998). However, another report has concluded that infection of ethylene-insensitive soybeans with various pathogens has the opposite outcome (Hoffman *et al.*, 1999). In our experiments, intact plantlets or detached leaves and flowers from inducible ethylene-insensitive petunia mutants showed delayed and reduced disease symptoms. Obviously, the promotion or suppressive role of ethylene on disease symptoms during a plant–pathogen interaction depends on the plant variety and tissues, as well as the nature of the pathogen. This conclusion was also supported by Boller (1991) and Lund *et al.* (1998). Another considered reason is that the inducible promoter system can regulate the expression of *etr1-1* at will by the addition of the inducer. As ethylene signalling is involved in multiple aspects of plant development, the mutation of the ethylene receptor can lead to other undesirable physiological effects and defective growth (Knoester *et al.*, 1998). The state of the induced *etr1-1* expression in such a short time in petunia, i.e. 8 days on intact plantlets and

3–4 days on detached leaves or flowers, determines the normal phenotype, and may make petunia more tolerant to grey mould development than the other model plants with constitutive expression of *etr1-1*.

In our previous research, we observed that the *etr1-1* gene was more strongly expressed in the E9 line relative to the E7 line (Wang *et al.*, unpublished data). Gallie (2010) demonstrated that the extent of ethylene insensitivity was caused by the level of *etr1-1* expression. Reduced or delayed disease symptoms in plantlets, leaves and flowers in the E9 line relative to the E7 line supports the conclusion that the reduced disease phenotype is related to a higher level of the *etr1-1* expression.

A notable point is that about 50% of the inoculated samples did not become infected when *etr1-1* expression was induced in either line. Although the lack of a wound may have contributed to the reduction in infection, as *B. cinerea* infects through wounds or natural openings on diverse plant organs (Holz *et al.*, 2004), the consistent incidence of disease in the two lines suggests that the

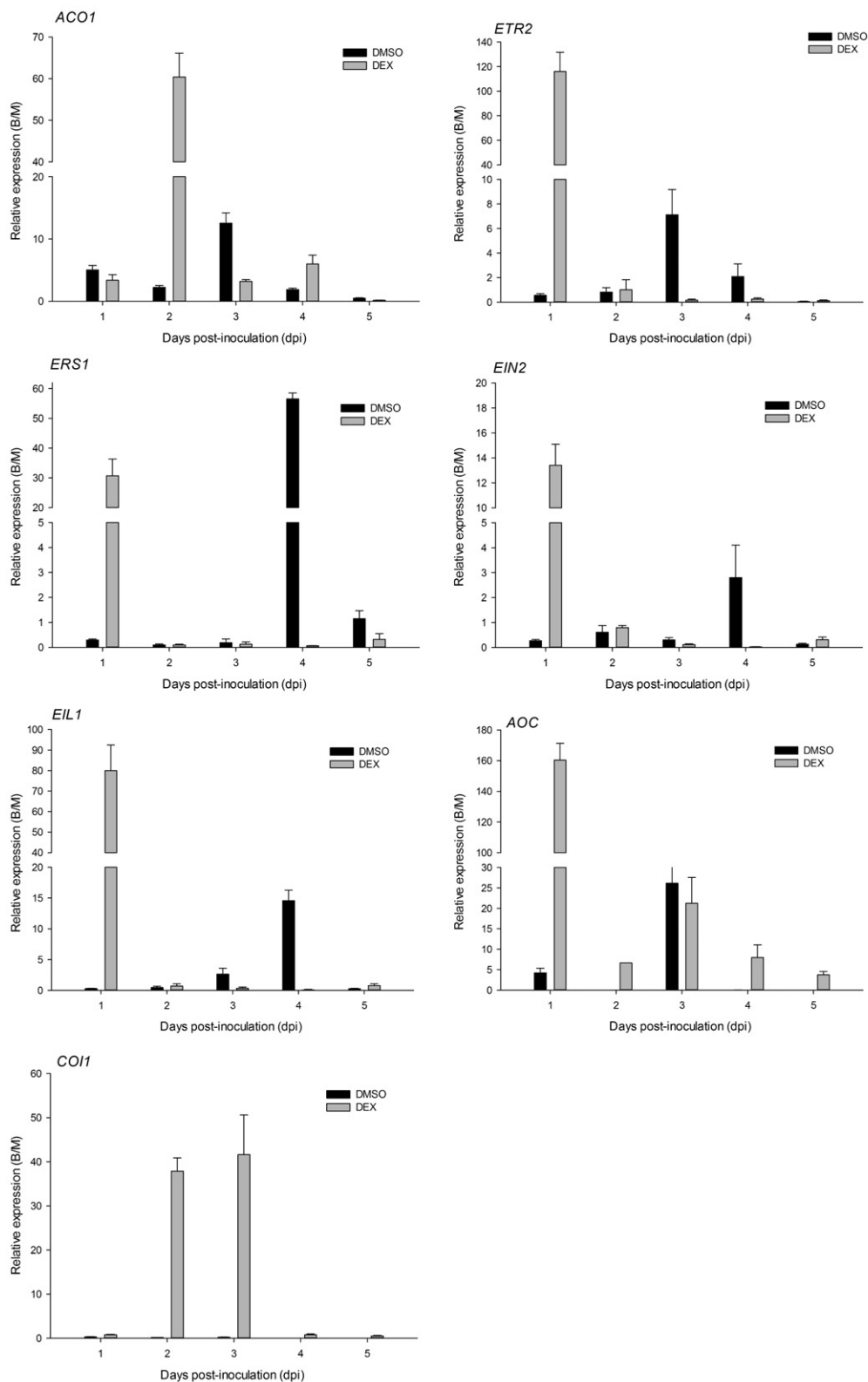


Fig. 6 Expression patterns of ethylene and jasmonate (JA) pathway genes in *Botrytis cinerea*-inoculated plantlets relative to expression in mock-inoculated E9G. B/M, *Botrytis*/mock. See text for gene definitions.

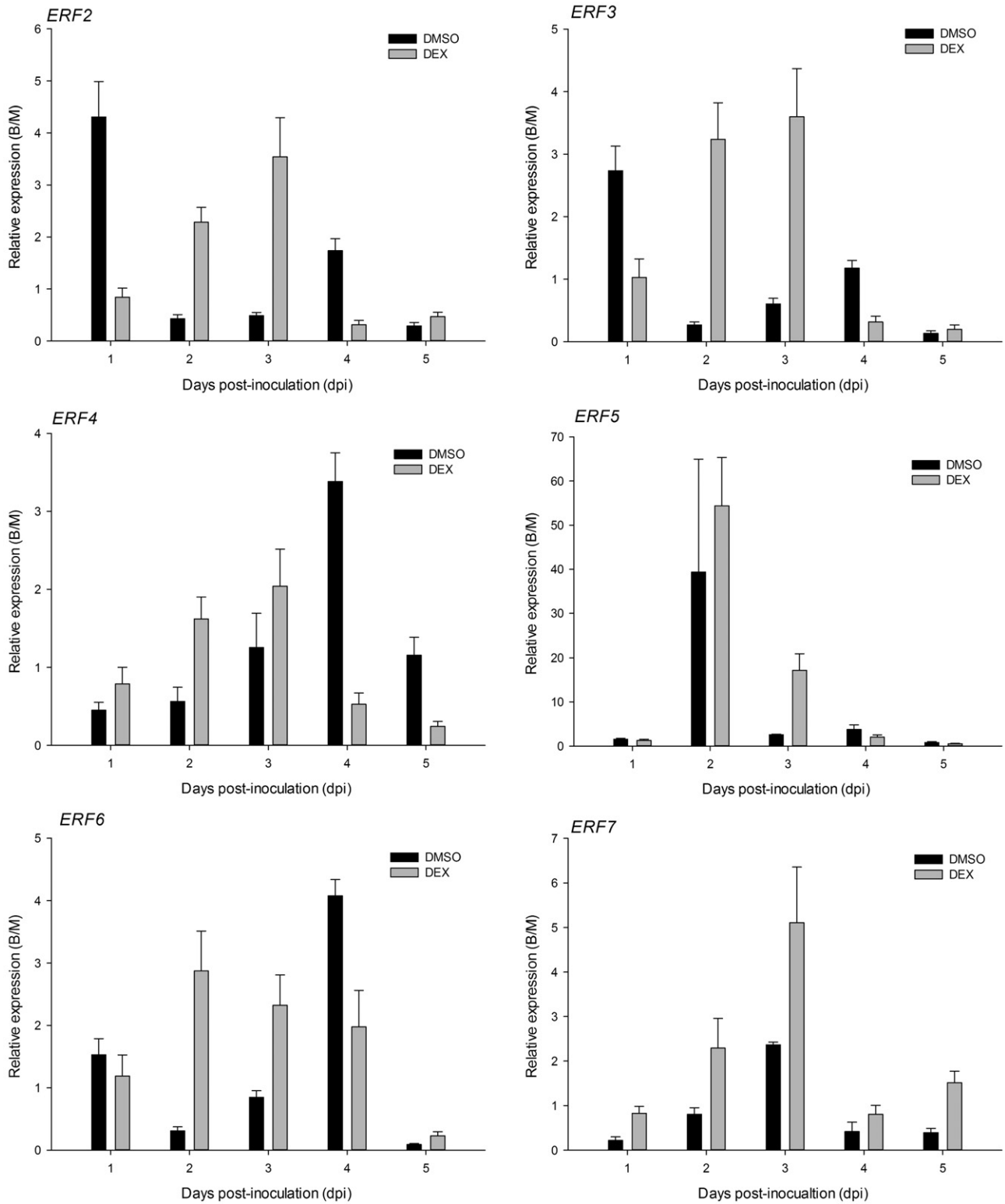


Fig. 7 Expression patterns of petunia ethylene response factor (*PhERF*) genes in *Botrytis cinerea*-inoculated plantlets relative to expression in mock-inoculated E9G. B/M, *Botrytis*/mock.

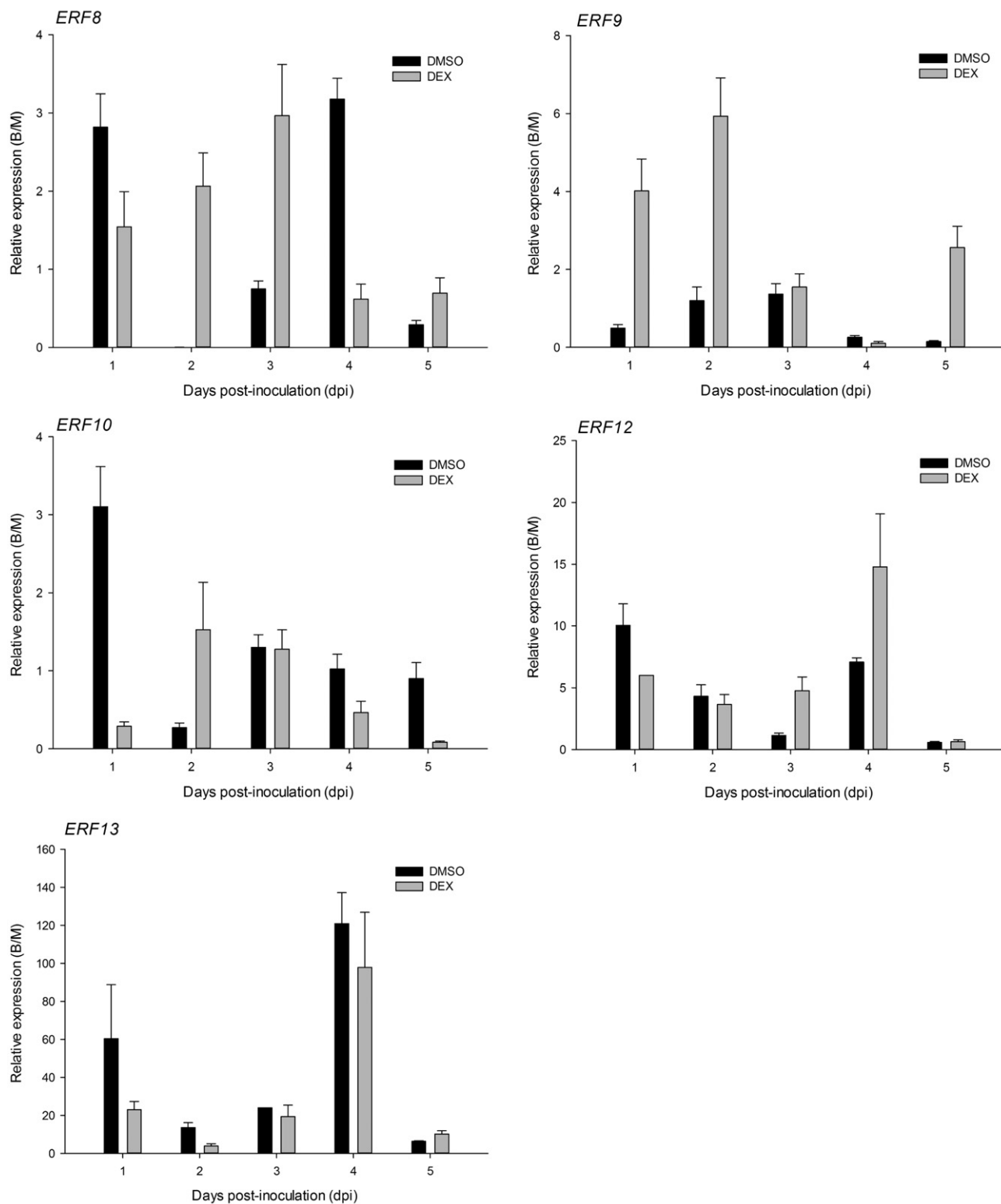


Fig. 7 Continued

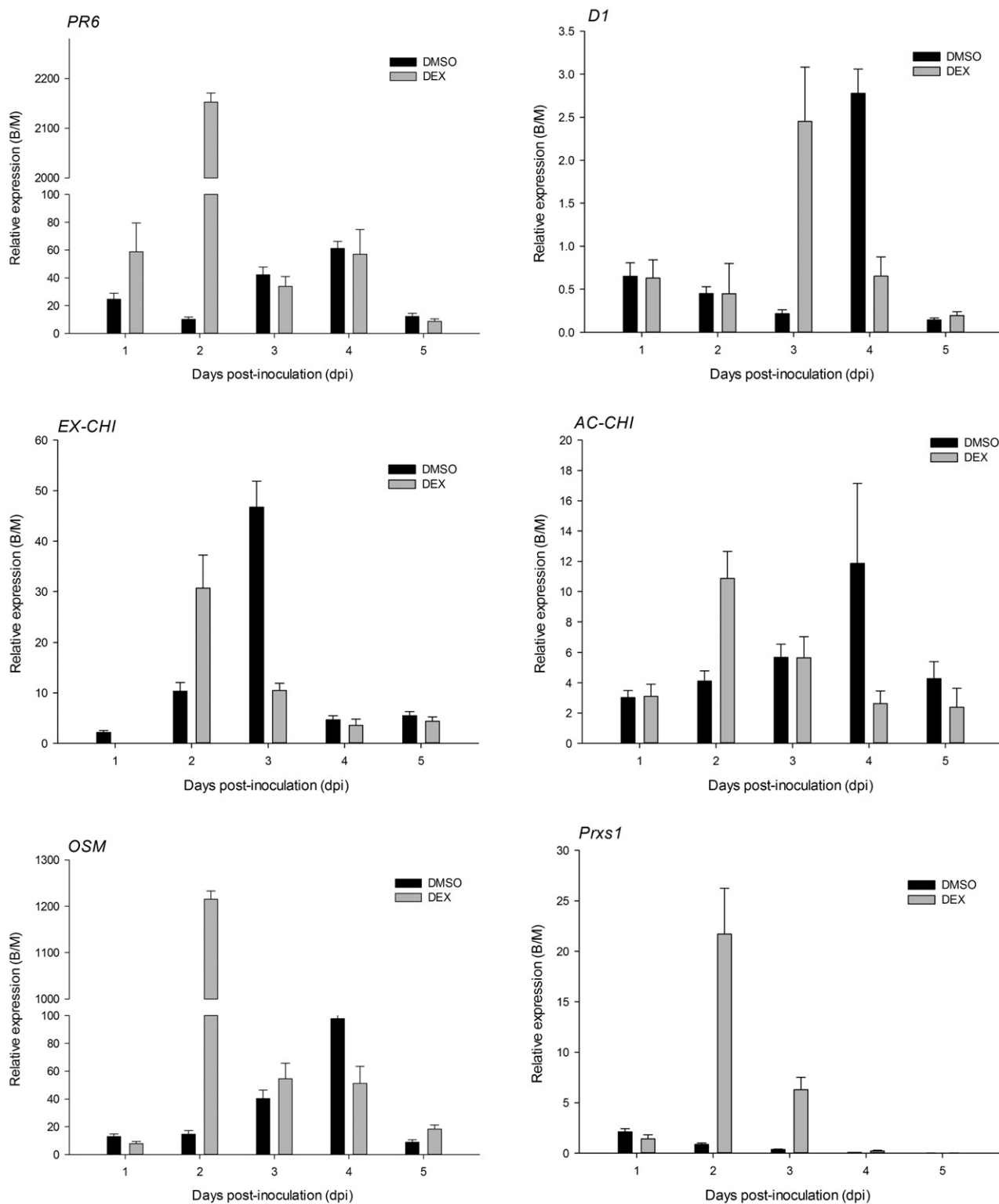


Fig. 8 Expression patterns of defence-related genes in *Botrytis cinerea*-inoculated plantlets relative to expression in mock-inoculated E9G. B/M, *Botrytis*/mock. See text for gene definitions.

extent of pathogen resistance of the detached leaves of *etr1-1*-expressing plants is important for the response of petunia to *B. cinerea*.

Ethylene regulates PCD induced by the pathogen

During the colonization of host tissue, the lower chlorophyll loss rate and increased MDA levels resulting from *B. cinerea* infection of DEX- rather than DMSO-treated leaves demonstrates that ethylene responses play a role in delaying necrosis induced by *B. cinerea*.

Botrytis cinerea boosts senescence through the production of ethylene (Cristescu *et al.*, 2002), which promotes leaf senescence, together with age-related factors (Grbić and Bleeker, 1995), as well as fruit ripening and flower senescence (Klee and Clark, 2010). Apart from ethylene, *B. cinerea* is known to produce abscisic acid (ABA) (Sharon *et al.*, 2007), a known inducer of senescence (Lim *et al.*, 2007). Infection with *B. cinerea* induced the senescence-associated gene *SAG12* (Swartzberg *et al.*, 2008), a member of a group of genes with senescence-specific patterns of transcript accumulation (Lohman *et al.*, 1994; Thomma *et al.*, 1999). In our experiments, it is worth mentioning that the expression of *PhCP2* and *PhCP4* was not related to the senescence induced by *B. cinerea*, although *PhCP2* and *PhCP4* have been included in the group of genes up-regulated during senescence by phylogenetic analysis (Jones *et al.*, 2005). Other *PhCPs*, for example *PhCP3*, which was grouped with genes up-regulated by drought and wounding (Jones *et al.*, 2005), or *PhCP6* and *PhCP7*, genes up-regulated by wounding, infection or PCD (Asp *et al.*, 2004; Lidgett *et al.*, 1995; Schmid *et al.*, 1999), did not exhibit senescence-associated expression in response to *B. cinerea*. Higher expression of *PhCP10*, which is very similar to *SAG12* (Jones *et al.*, 2005), and *PhCP8* was found at 2 and 3 dpi on DMSO- rather than DEX-treated plantlets. Together with the visible senescence symptoms, our results show that *PhCP8* and *PhCP10* are senescence-associated genes and induced ethylene insensitivity in petunia retards the senescence caused by *B. cinerea*.

The independent roles of ethylene and JA on the plant response to pathogen

Were other ethylene receptors involved in the reduction in disease symptoms in *etr1-1*-expressing petunias? The expression of *ETR2*, *ERS1*, *EIN2* and *EIL1* was inhibited, together with the induced expression of *etr1-1*, after infection. Typically, plants produce high levels of ethylene when challenged by pathogens, especially necrotrophs, such as *B. cinerea* (Broekaert *et al.*, 2006; van Loon *et al.*, 2006; Tsuchisaka *et al.*, 2009). In addition, microorganisms, such as *B. cinerea*, can produce ethylene (Cristescu *et al.*, 2002). The gene expression of *ACO* was observed on both DEX- and

DMSO-treated leaves, which supports the conclusion that the ethylene levels produced by either the petunia or the pathogen during the interaction are not sufficient to affect the expression and function of ethylene receptors by a feedback mechanism. These results show that the induced expression of *etr1-1* using the GVG promoter totally prevents the ethylene signalling pathway from participating in the plant defence response to *B. cinerea*.

More interestingly, the expression of *PhCO11* was almost entirely suppressed when the ethylene signalling pathway was activated, although the biosynthesis of JA was predicted because of the expression of *PhAOC*. However, transcriptional accumulation of *PhCO11* was detected when the ethylene pathway was blocked. The *PhERFs* showing maximum expression levels at 3 dpi were *PhERF2*, *PhERF3*, *PhERF4*, *PhERF7* and *PhERF8*; this was the same expression pattern as for *PhCO11* in DEX-treated leaves. The coincidence of expression patterns between these *PhERFs* and *PhCO11* suggests that JA-independent ethylene induces or regulates the expression of these *ERF* genes. In addition, the expression of *ETR2*, *ERS1*, *EIN2* and *EIL1* was activated without DEX treatment after inoculation. The maximum expression levels of *EIN2* and *EIL1*, which play a central role in the transduction of the ethylene signal to downstream targets (Shibuya *et al.*, 2004), corresponded to the peak levels of *PhERF4*, *PhERF6*, *PhERF8* and *PhERF13* at 4 dpi; this led us to conclude that these four *ERF* genes were activated through the ethylene signalling pathway during the interaction between petunia and *B. cinerea*. Consequently, we propose that: (i) when petunia was assailed by *B. cinerea*, ethylene and JA independently established signal transduction; and (ii) *PhERF4* and *PhERF8* were activated through either the JA or ethylene pathway. These two genes appeared to perform a different function from *Arabidopsis* *ERF1* and *ORA59*, which are integrators of the JA and ethylene signalling pathways (Leon-Reyes *et al.*, 2010; Lorenzo *et al.*, 2003; Pré *et al.*, 2008). The peak of expression of these two genes occurred 1 day earlier in the JA pathway than in the ethylene pathway. This result might be related to the delayed senescence induced by the expression of *etr1-1*. This provides a research direction for the future to investigate the detailed functions of *PhERF4* and *PhERF8* in JA and ethylene mutants.

The expression of plant genes encoding for PR proteins was assessed during the petunia–*B. cinerea* interaction. In contrast with other species, for example, tomato infected by bacterial wilt and canker pathogen, no significant differences in the expression of defence-related genes were found in comparison with the ethylene-insensitive mutant *Nr* and wild-type plants (Balaji *et al.*, 2008). In our experiments, the expression of *PR6*, *OSM* and *Prxs1* was increased when *etr1-1*-expressing plants encountered *B. cinerea*. The JA-dependent expression of *PR6*, *OSM* (*PR5*) and *Prxs1* genes has been reported previously (Buzi *et al.*, 2004; Hondo *et al.*, 2007; Xu *et al.*, 1994; Zahn *et al.*, 2005); 35-, 12- and 11-fold greater expression of these three genes, respectively, were

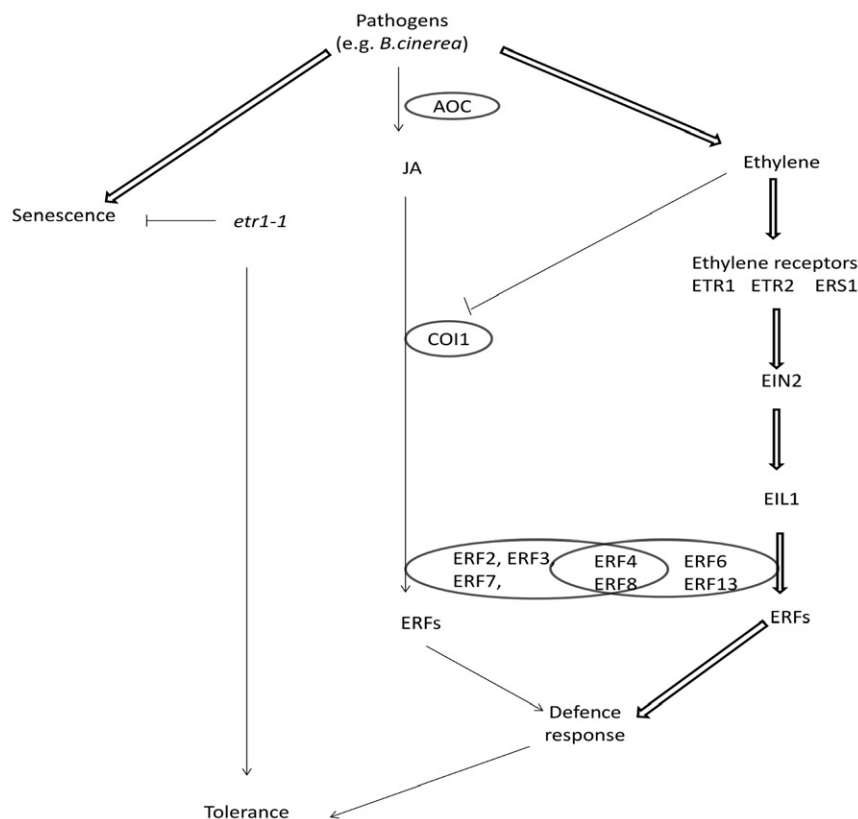


Fig. 9 Model of ethylene/jasmonate (JA) independent pathways of petunia response to pathogens. When petunia encounters pathogen, the biosynthesis and activation signalling of the ethylene pathway are induced (wide arrows). Subsequently, some petunia ethylene response factor (*PhERF*) genes, such as *PhERF4*, *PhERF6*, *PhERF8* and *PhERF13*, are activated in order to induce plants to launch the basal defence response, although finally programmed cell death and senescence occur. Otherwise, the ethylene signalling pathway is completely blocked by induced *ethylene-insensitive-1-1 (etr1-1)* expression, and the biosynthesis and consequent signalling of the JA pathway are induced by *B. cinerea* infection (narrow arrows). Some *PhERFs*, such as *PhERF2*, *PhERF3*, *PhERF4*, *PhERF7* and *PhERF8*, are activated at the transcriptional level. In turn, the transcriptional accumulation of defence-related genes, together with delayed senescence (narrow arrows) makes ethylene-insensitive petunia tolerant to the pathogen to some extent (narrow arrows). See text for gene definitions.

seen in DEX-treated relative to DMSO-treated leaves. These results further suggest that the JA pathway was active during the petunia response to *B. cinerea* infection when *etr1-1* was induced. The peak expression levels of these genes were seen at 3 dpi, followed by the expression of *PhCOI1* and *PhERF2*, *PhERF3*, *PhERF4*, *PhERF7* and *PhERF8*. The coincidence of the gene expression profiles of *PhCOI1*, *PhERFs* and JA-associated defence-related genes suggests that the JA signalling pathway is responsible for the delayed disease symptoms and decreased infection by *B. cinerea*. However, in DMSO-treated plantlets, the peak expression of all of the tested defence-related genes, with the exception of *Prxs1*, was 4 dpi, which is consistent with the peak level of *PhEIL1* and other *PhERFs* (*PhERF4*, *PhERF6*, *PhERF8* and *PhERF13*). These results suggest that ethylene is involved in regulating the petunia basal adaptation response to *B. cinerea*. This conclusion is supported by the higher expression levels of *EX-CHI* and *AC-CHI*, both of which are ethylene response genes (Rakwal *et al.*, 2004), in DMSO-treated leaves relative to DEX-treated leaves.

In conclusion, our findings provide a model of ethylene/JA-independent responses when petunia plants are challenged by exposure to a necrotrophic pathogen, *B. cinerea* (Fig. 9). Petunia plants exposed to biotic stresses launch signalling events, including the biosynthesis and signalling transduction of ethylene, completely independent of the JA pathway, which activates the downstream expression of *PhERF4*, *PhERF6*, *PhERF8* and *PhERF13*.

These pathways then promote plant basal defence responses, although, finally, plant death occurs. When the ethylene signalling pathway is blocked, the biosynthesis and signalling transduction pathways of JA are activated, which trigger the expression of *PhERF2*, *PhERF3*, *PhERF4*, *PhERF7* and *PhERF8*, thereby increasing the expression of defence-related genes. The specific characteristics of ethylene insensitivity and the defence response activated by the JA pathway are responsible for petunia tolerance to this pathogen.

EXPERIMENTAL PROCEDURES

Plant material

Petunia seedlings were obtained by germinating seeds in Murashige and Skoog (MS) medium. The T₂ seeds of transgenic *Petunia × hybrida* cv. Mitchell Diploid (MD) with GVG:*etr1-1* of E7 and E9 lines, which showed different expression levels of *etr1-1* after DEX induction in our previous research, were first surface sterilized in 15% bleach for 20 min and then in 70% ethanol for 45 s, followed by three washes with sterile distilled water. After placing the seeds (20 seeds/plate), the plates were kept at room temperature under continuous low fluorescence light (~40 μmol/m²/s) for 21 days. The young seedlings were then carefully removed from the MS medium and placed in 10-cm pots. Plants were grown in a growth chamber with a 16-h photoperiod, at 25 °C in the light period and 22 °C in the dark period. Plants were fertilized once a week with nitrogen at

300 mg/L from 15N-5P-15K Cal Mag (Peters soluble fertilizer, The Scotts Co., Marysville, OH, USA).

Plant treatments

DEX (Sigma-Aldrich, St. Louis, MO, USA) was stored as a 10 mM solution in 60% (v/v) dimethylsulphoxide (DMSO) (Sigma-Aldrich) at -20°C . DEX was added to vase solution (Chrysal, Miami, FL, USA) to achieve induction. Unless otherwise stated, 30 μM DEX was used (Craft *et al.*, 2005).

Intact plants, detached leaves and flowers were subjected to different treatments with 30 μM DEX or without the chemical inducer, i.e. 0.18% (v/v) DMSO, as a control. Eight-week-old plants were irrigated with the chemicals for 4 days before inoculation. The irrigation solution was replaced every 4 days after inoculation according to plant requirements and previous reports (Wielopolska *et al.*, 2005). The fourth leaves from the top were collected and placed in boxes containing 0.6% agar medium. The inducers were painted 2 days before inoculation and painting was maintained during the inoculation period when needed. Flowers from T₃ plants of selected lines were harvested when fully open, but before anther dehiscence. Flowers were placed in 2-mL tubes containing chemicals 2 days before inoculation.

Inoculation assays

Botrytis cinerea Pers.:Fr. strain B05.10 was grown on potato dextrose agar plates (Sigma-Aldrich). The conidial suspension was filtered through a cloth mesh (Cantu *et al.*, 2008). After removal of the supernatant and resuspension of the conidia in sterile distilled water, an inoculation suspension was prepared containing 1.0×10^6 conidia/mL with 10 mM glucose and 6.7 mM potassium phosphate (pH 6.0) (Audenaert *et al.*, 2002; Díaz *et al.*, 2002). Conidia were pregerminated without shaking for 2 h in the inoculation suspension at 22°C (Asselbergh *et al.*, 2007; Díaz *et al.*, 2002).

For the leaf disc and flower assays, two 10- μL droplets were used to inoculate each petunia leaf, which was incubated at 25°C under dark conditions. Disease severity and disease incidence were evaluated and analysed every day after inoculation. At least 80 inoculation drops were evaluated for each treatment. Detached flowers were inoculated under the same induction conditions as the detached leaves. The numbers of healthy flowers, expanding lesions per flower and lesion expansion were investigated after 3 days and during the period from 2 to 3 days after inoculation. At least 16 droplets were analysed for each treatment.

For intact plant assays, 300 μL of the conidial suspension were sprayed on the leaves. Inoculated plants were grown in a sealed light transmittance box with a pinhole foil in a 16 h/8 h ($\sim 40 \mu\text{mol}/\text{m}^2/\text{s}$) photoperiod growth chamber, at 25°C in the light period and 22°C in the dark period. The same indices as detached flowers were performed on the fifth day and in the period between 5 and 6 days after inoculation. At least 10 plants were assessed in each treatment.

Chlorophyll measurements

Chlorophyll in intact plant leaves and detached leaves was measured by SPAD (Konica, Osaka, Japan) every day after inoculation (Coste *et al.*,

2010). Three spots per leaf were selected to read. At least 45 and 40 leaves from the *in vivo* and *in vitro* investigations, respectively, were evaluated for each treatment.

Lipid peroxidation and antioxidant enzyme analysis

MDA activity was assayed to assess lipid peroxidation in approximately 0.2 g (fresh weight, FW) of detached petunia leaves using a modified thiobarbituric acid (TBA)-MDA assay (Hodges *et al.*, 1999). Approximately 0.5 g FW of detached leaves were homogenized in a prechilled mortar and pestle in liquid nitrogen with 0.5 g of polyvinylpyrrolidone, and incubated in enzyme extract buffer containing potassium phosphate buffer (pH 7.5) for 0.5 h at 4°C . The extract buffer containing ground tissue was then centrifuged (Eppendorf, Hamburg, Germany) at $13\,362 \times g$ for 20 min at 4°C . The supernatant was divided into two identical aliquots. Each 0.1-mL supernatant was supplemented with the reaction buffer for SOD or CAT analysis, according to Hodges and Forney (2000). All enzymes were assayed spectrophotometrically at 25°C with UV-VISO 2450 (Shimadzu, Kyoto, Japan).

RNA extraction and qRT-PCR analysis

Total RNA from separate, duplicate petunia leaves *in vivo* was isolated using Trizol (TaKaRa, Dalian, China). One microgram of total RNA was reverse transcribed using the PrimeScript RT reagent with the gDNA Eraser Kit (TaKaRa), according to the manufacturer's instructions. In brief, 1 μg of total RNA was cleared of DNA with 2 μL of $5 \times$ gDNA Eraser Buffer and 1 μL of gDNA Eraser for 2 min at 42°C . The 10- μL reaction volume was reverse transcribed at 37°C for 15 min in a 20- μL reaction volume containing 4 μL of $5 \times$ PrimeScript Buffer2, 1 μL of PrimeScript RT Enzyme Mix I and 1 μL of RT Primer Mix. The reaction was stopped by incubation at 85°C for 5 s. One microlitre of diluted $5 \times$ cDNA was used as the template for PCR amplification using SYBR GREEN Taq (TaKaRa). Specific primers were designed by the PRIMER 3 program and are listed in Table S1 (see Supporting Information). Each gene was tested in a volume of 20 μL . Amplifications were performed using an Applied Biosystems 7300 system (Applied Biosystems, Shanghai, China) with the following procedure: 2 min at 95°C , followed by 40 cycles of 45 s at 95°C , 30 s at 60°C and 45 s at 72°C . Fluorescence was measured at the end of each cycle. Analysis of the melting curves provided evidence for the absence of nonspecific products and primer dimers. For data analysis, average threshold cycle (C_T) values were calculated for each gene of interest, on the basis of three independent biological samples as indicated (Balaji *et al.*, 2008), and were normalized and used to calculate the relative transcript levels as described elsewhere (Pfaffl, 2001). 26SrRNA was used as an internal standard for normalization (Balaji *et al.*, 2008).

Statistical analyses

All statistical analyses were performed using the SPSS package (Version 16.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed for experiments with one independent variable. Duncan's test was used as the *post hoc* test if significant differences were found.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 The representative phenotype of dexamethasone (DEX)- and dimethylsulphoxide (DMSO)-treated E7H petunias inoculated by *Botrytis cinerea*.

Table S1 Gene-specific primers for real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR).