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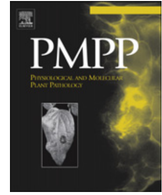

Arabidopsis ethylene receptors have different roles in Fumonisin B_1-induced cell death

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ABSTRACT

Ethylene is a central signalling agent in mediating plant defence against pathogens. Mutations to the ethylene receptor ETR1 have been shown to alter susceptibility of plants to mycotoxin-induced cell death. Using Fumonisin B_1 (FB_1) to induce cell death, we demonstrate that the receptor mutant *ein4-1* has a reduced rate of necrosis, potentially due to an upregulation of *ETHYLENE RESPONSE FACTOR1*. Mutations in other ethylene receptors differentially affected the expression of genes in the jasmonic and salicylic acid defence pathways. Together these data indicate that ethylene receptors do not have redundant roles in mediating FB_1-induced cell death.

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1. Introduction

Plants have well developed systems to recognise self versus non-self and to mount defence against different pathogens. A number of stressors can elicit a defence response in the plant, including herbivory, mechanical damage and other abiotic stresses. Once the stress is detected within the plant, programmed cell death can be activated by the coordinate signalling of at least three defence pathways: the salicylic acid pathway (SA), the jasmonic acid pathway (JA) and the ethylene pathway. Expression levels of key genes from each pathway have been used to monitor the intensity of the defence response. For example, expression of *PATHOGENESIS RELATED PROTEIN1 (PR1)* is used to determine the activation of the SA pathway [1–3] while *VEGETATIVE STORAGE PROTEIN1 (VSP1)* and *ETHYLENE RESPONSE FACTOR1 (ERF1)* are used for the JA and ethylene pathways, respectively [4–6]. Other genes such as *PLANT DEFENSIN1.2 (PDF1.2)* are activated concomitantly by ethylene and JA and can be used to follow crosstalk between the pathways [4]. In all cases, JA, ethylene and SA positively regulate downstream signalling and impairment to any of these pathways via mutation or chemical blockage leads to increased pathogen susceptibility.

Ethylene is an important mediator in response to necrotrophic pathogen attack [7], but there are conflicting results with regard to the exact role of ethylene in mediating cell death. While it has been found that exposure to exogenous ethylene can increase disease symptoms such as increased rate of necrosis and spread of pathogens [7–9], it has also been shown that ethylene must be present for resistance to other pathogens such as *Botrytis cinerea* and *Erwinia carotovora* [10,11]. Ethylene is a gaseous plant hormone that is sensed by a set of five receptors in *Arabidopsis* (ETR1, ERS1, ETR2, ERS2 and EIN4) that are localized to the endoplasmic reticulum [12], and in the case of ETR1, to the golgi apparatus [13]. The receptors form hetero or homodimers with each other that, in the absence of ethylene, associate with CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) [14–16]. Through an unknown mechanism, the receptors maintain the activity of CTR1 in the absence of ethylene, which actively represses the ethylene pathways [17,18]. When ethylene is present, it binds to the transmembrane portion of the receptor through a copper co-factor [19]. The binding of ethylene causes CTR1 to be inactivated, thereby relieving CTR1's repression on the ethylene response pathways through activation of EIN2, a downstream signal relay for the ethylene pathways [16,20]. The ETR1 receptor, and possibly the ERS1 receptor, can also positively control ethylene responses through a CTR1 independent pathway based on their histidine kinase activity [21]. Mutations that prevent ethylene from binding to a given receptor (*etr1-1*, *etr2-1*, *ers1-1*, *ers2-1*, *ein4-1*) cause plant-wide ethylene insensitivity [22–26]. These mutations are known as gain-of-function mutations because the ethylene receptors activate CTR1 in the absence of ethylene. The majority of current research into the role of ethylene perception on cell death during pathogen attack has focused on one mutant, *etr1-1* [27–30], likely due to the view that the five ethylene receptors from *Arabidopsis* act redundantly [19,20,24]. This concept of the ethylene signalling pathway has recently been revised due to new findings which show that, in limited cases, ethylene receptors can signal through

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a non-CTR1 dependent pathway [21,31] and that the receptors can form heterodimers in vivo rather than exclusively homodimers as previously thought [12]. This may mean that ethylene receptors do not have redundant functions. Given the diverse, and sometimes contradictory, effects of ethylene in mediating pathogen induced cell death it is important to analyze the individual roles of each ethylene receptor.

Necrotrophic pathogens, such as *Fusarium verticillioides* (formerly *Fusarium moniliforme*), produce mycotoxins such as Fumonisin B_1 (FB_1) that kill plant cells in order that the fungus may feed off of the dead cells [32–36]. FB_1 blocks sphingolipid metabolism through competitive inhibition of ceramide synthase and causes rapid cell death in plant cells [37]. FB_1 is important in human epidemiology as consumption of contaminated crops is thought to be correlated with a high incidence of neural tube defects in developing countries [38]. FB_1 can be infiltered directly into plant leaves to mimic colonization of *F. verticillioides* for research on host pathogen interactions [1]. Using FB_1, we mimicked fungal induced cell death in all five *Arabidopsis* gain-of-function ethylene receptor mutants (*ers1-1*, *ers2-1*, *etr1-1*, *etr2-1* and *ein4-1*). We analyzed differences in the rate of cell death between the mutants, the role of ethylene signalling in ethylene receptor expression as well as the effect of ethylene insensitivity on the SA and JA pathways in cell death was also investigated. From these data, we demonstrate that there are specific roles for some ethylene receptors in mediating FB_1-induced cell death.

2. Materials and methods

2.1. Plant growth conditions

Arabidopsis seeds, obtained from the Arabidopsis Biological Resource Center, were stratified for 4 days at 4 °C and grown under long-day conditions (16 h light/8 h dark) at a light intensity of 130–190 μE m^{-2} s^{-1} at the rosette level at 21 °C in Econair AC-60 growth chambers. The mutants *ers2-1*, *etr1-1*, *etr2-1* and *ein4-1* were in the Columbia background, and were therefore compared to it as a control. The mutant *ers1-1* was in the Nossen background, therefore all results from *ers1-1* were compared to the Nossen ecotype as the wildtype control. Infiltration and plate experiments were performed at least 4 separate times to ensure reproducibility and significance of the results presented. Biological replicates were pooled from independent experiments to ensure reproducibility.

For growth on agar plates supplemented with FB_1, *Arabidopsis* seeds were sterilized then stratified at 4 °C for 3 days before being plated on Murashige and Skoog basal salt mixture (Sigma, Oakville, ON, Canada) adjusted to pH 5.7–5.8, 0.8% (w/v) agar. Plates were supplemented with FB_1 (Sigma, Oakville, ON, Canada) to a final concentration of 0–1 μM. Seedlings were grown under the same conditions listed above for 10 days at which point images were taken of representative amounts of growth under each test condition. Four replicates of 25 seeds each were tested at each concentration of FB_1.

2.2. FB_1 treatment and microscopic analysis

Fumonisin B_1 treatment was performed on four week old rosette leaves. Approximately 100 μL of 5.0 μM FB_1 or 0.14% methanol as a control were infiltered into the leaves from the abaxial surface using a syringe. In total, 150 plants of each mutant and for each wildtype ecotype were infiltrated with the FB_1 solution, and an equal number of plants infiltrated for control. Samples (1 cm diameter leaf discs) were taken every 12 h post-infiltration for 96 h. Samples were photographed or frozen immediately at −80 °C for RNA extraction ($n = 6$ per time point). Samples were also taken for microscopic analysis, cleared by boiling in lactophenol ($n = 4$ leaves per time point). Phenolic accumulation was determined by autofluorescence while callose deposition was rendered after staining samples with aniline blue (0.03% aniline blue in PBS for 10 min) and pictured using a Carl Zeiss Axioplan Fluorescent microscope (Carl Zeiss, Germany).

2.3. Chlorophyll analysis

Six samples taken every 12 h were analyzed for chlorophyll loss as per United States Environmental Protection Agency standard operating procedure (Protocol #2030, 1994). In short, 1 cm diameter leaf discs were extracted in 80% acetone and chlorophyll content was measured by a SPECTRAmax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) at 645 nm and 663 nm to determine the concentrations of chlorophyll A versus B.

2.4. Expression profiling

Expression analysis of ethylene receptors and *VSP1*, *PR1*, *PDF1.2* and *ERF1* was performed using quantitative PCR (qPCR). Total RNA was extracted using the Qiagen RNeasy Plant Extraction Kit (Mississauga, Ontario) and cDNA synthesis reverse transcribed with iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Mississauga, Ontario). *Ubiqutin 10* was used as an internal reference gene as it has been found to be stably expressed in microarray studies [39]. To ensure that this was a proper control, we tested the expression of this gene in each tested condition. It was determined that it was stably expressed in both control and test conditions. All primers were tested for a linear dose:response. Only primers that gave a linear dose:response were used in this study. Primers were also tested, via melt curve analysis and gel electrophoresis, to ensure that only one product was produced. Primers used for the amplification of *UBQ10* were 5′-GTCCTCAGGCTCCGTGGTG-3′ (forward) and 5′-TGCCATCCTCCAACTGCTTTC-3′ (reverse).

The following primers were used for ethylene receptor genes:

5′-TGGATTGAGAGCGATGGTCTTGG-3′ (forward) and
5′-GAATGGCTGGAACTTTCGGTATGC-3′ (reverse) for *ETR1*,
5′-GTGGTTATCCACGATGTAACTGCGA-3′ (forward) and
5′-ATGGAACATTGGAACAACTCAC-3′ (reverse) for *ETR2*,
5′-CAAGCTATGCACATACCTCATTC-3′ (forward) and
5′-TCTCCATTTTCTTGCACCATCGGTT-3′ (reverse) for *ERS1*,
5′-TGGTTTCACCTACGGGCCTCACTG-3′ (forward) and
5′-AAGAGAGTGACTAACGAGAGTGCC-3′ (reverse) for *ERS2*, and
5′-AGAGGCAGAAGGAAATGAGTGTGCA-3′ (forward) and
5′-TGAGTCAAATGCATCTCGGTT-3′ (reverse) for *EIN4*.

The defence gene primers were:

5′-GCTCTTGTTCTCTTTGCTGCTTTCGACG-3′ (forward) and
5′-GCATTACTGTTTCCGCAAACCCCTGAC-3′ (reverse) for *PDF1.2*,
5′-GACTCATACACTCTGGTGGGCC-3′ (forward) and
5′-CTC GCT AAC CCA CAT GTT CAC GG-3′ (reverse) for *PR1*,
5′-CGCCAAAGGACTTGCCCTAAAG-3′ (forward) and
5′-CGGGGCTGTGTTCTCGGTCCC-3′ (reverse) for *VSP1*,
5′-GAGGCGTAAGACGACGGCCATGG-3′ (forward) and
5′-GCTAAAGCCGCCTCTTCCGCGC-3′ (reverse) for *ERF1*.

MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, Ontario) was used to measure the 3–4 biological replicates for the expression levels of ethylene receptor genes and the Qiagen Quantitec SYBR Green kit was used to analyze 3 biological replicates each for the expression levels of defence related genes (Qiagen, Mississauga, Ontario). Manufacturers' instructions for standard cycling procedures were followed in each instance. All values were normalized using the internal control (*UBQ10*) and then expressed as fold induction or repression of each gene during FB_1 treatment as compared to gene expression during mock treatment.

2.5. Statistical analysis

Comparison of expression levels between all mutants and wildtype were analyzed statistically using a *T*-test for two samples assuming unequal variance with $p < 0.05$.

3. Results

3.1. Ethylene receptors differentially mediate FB_1-induced cell death

During colonization of a plant by a pathogen, the progress of the disease symptoms can be followed using a variety of developmental indices. Macroscopically, death can be quantified by timing the appearance of necrotic lesions and the rate of chlorosis can be measured by chlorophyll loss. Necrotic lesions developed in 50% of *ers1-1*, *ers2-1* and *etr2-1* plants at the same time (48 h) as their respective wildtype backgrounds (Fig. 1a). In contrast, rapid cell death was observed in *etr1-1*, where lesions in the majority of plants were seen as early as 24–36 h. In *ein4-1* there was little to no damage caused FB_1 infiltration in most *ein4-1* plants infiltrated at 96 h. When seeds were grown on MS media containing different concentrations of FB_1, it was found that the mutants responded similarly to when leaves were infiltered with the toxin (Fig. 1b). It was found that over 50% of the *etr1-1* mutant was stunted and exhibited high levels of chlorosis at concentrations as low as 0.01 μM FB_1 while *ers1-1*, *ers2-1*, *etr2-1* and wildtype did not display significant stunting nor cell death until 0.1–0.5 μM FB_1 (Fig. 1b). Finally, over 50% of the *ein4-1* plants were still alive on 0.5 μM FB_1, although no true leaves were produced during the test duration of 10 days while wildtype and *etr1-1* germinated and died within 5 days of germination. The *etr1-1*, *etr2-1*, *ers2-1* and *ein4-1* mutants were in the Columbia-0 background while the *ers1-1* mutant was created in the Nossen background. All analyses used both wildtype backgrounds.

To indirectly evaluate the quantity of cell death, leaf discs of wildtype and the ethylene receptor mutants were analyzed for chlorophyll loss due to FB_1-induced cell death (Fig. 2). Chlorophyll loss has been found to play an important role in disease response [40], has been used to indirectly quantify difference in cell death [41] and has been used in the analysis of ethylene's involvement in FB_1-induced cell death in tomato [42]. It was found that *ers1-1*, *ers2-1* and *etr2-1* had similar rates of chlorophyll loss when compared to wildtype. In contrast, the degradation of chlorophyll was more rapid and more extensive in the *etr1-1* mutant and *ein4-1* showed little chlorophyll loss over the analyzed period. Quantification of cell death using this method supported our macroscopic observations that *etr1-1* had an increased rate of cell death while *ein4-1* displayed less cell death. The other ethylene receptor mutants responded like wildtype.

It is known that FB_1 induces a hypersensitive-like response (HR-like) [1]. As it was determined that there was a difference in the rate of cell death in two of the mutants tested, *etr1-1* and *ein4-1*, we investigated the accumulation of phenolics and callose [43], to determine if cell death was being activated in both mutants. It was determined, via phenolic autofluorescence and staining for callose, that accumulation of both types of compounds was induced in these mutant lines as well as in wildtype *Arabidopsis* (Fig. 3). A reduction in callose and phenolics were observed during early time points in *ein4-1*, but due to the variability between samples this difference from wildtype and *etr1-1* could not be assessed for significance.

3.2. Effect of FB_1 on defence gene expression

It is known that SA, JA and ethylene control the three main defence pathways in *Arabidopsis*. To ascertain whether these pathways were differentially affected by mutations to the ethylene receptors, we used quantitative PCR to follow the expression of representative defense genes in each pathway in plants grown on MS media containing 0.1 μM FB_1 as compared to plants grown on MS media. We wanted to get a more global look at the expression of defense genes throughout the plant therefore we used plants grown on media containing FB_1 rather than leaf discs infiltered with the toxin to ensure that FB_1 was sensed throughout the plant. At the concentration of FB_1 used in the media, all mutants tested had true leaves and were still growing but showed stunting due to FB_1. Expression of *VSP1*, which is activated by the JA pathway, was significantly activated in *ers1-1* (1 fold higher than wildtype) and *ers2-1* (8.6 fold over wildtype) (Fig. 4a) ($p < 0.05$). The expression of the *PR1* gene was used to follow induction of the SA controlled defence pathway (Fig. 4b). It was found that all five of the ethylene receptor mutants had significantly higher levels of *PR1* expression as compared to wildtype, although the degree of this induction was different in each mutant tested (*ERS1* – 29 fold; *ERS2* – 190 fold; *ETR1* – 68 fold; *ETR2* – 132 fold; *EIN4* – 115 fold). *ERF1* expression, used to mark induction of the ethylene pathway, was significantly higher in the *ein4-1* mutant (19 fold) (Fig. 4c). Conversely, *ERF1* expression was significantly repressed in *etr1-1* (1 fold) and *ers1-1* (8 fold) ($p < 0.05$). *PDF1.2* expression (Fig. 4d), which is influenced by both ethylene and JA, was repressed in *ein4-1* (2 fold) and *etr1-1* (5 fold), while levels in *ers1-1* (12 fold) and *ers2-1* (17 fold) were significantly higher than wildtype ($p < 0.05$). Therefore it was found that mutations to the ethylene receptors causing ethylene insensitivity differentially affected the expression of downstream defence related genes.

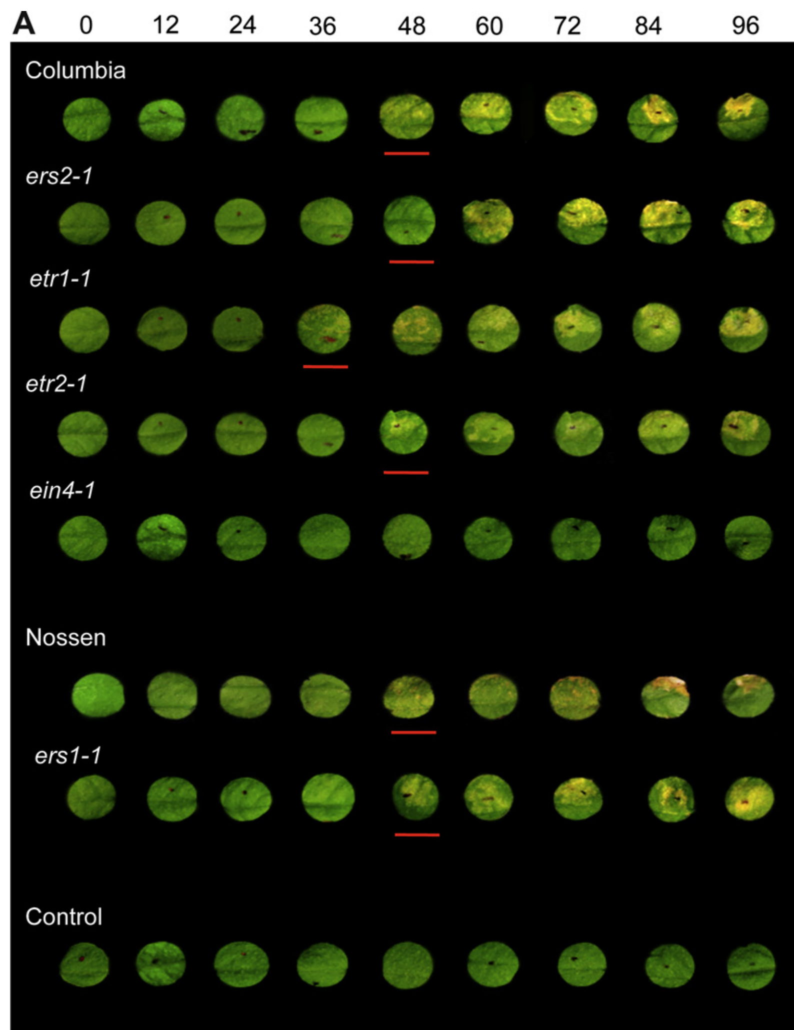
3.3. Ethylene insensitivity affects expression of ethylene receptors

Ethylene perception is believed to be a dynamic process where ethylene receptors influence their own levels through a feedback mechanism [44,45]. Using quantitative PCR we analyzed the degree of ethylene receptor gene induction or repression in wildtype and gain-of-function ethylene receptor mutants in the direct area around the infiltration of FB_1 infiltration at 48 h post-infiltration as compared to 0 h. The 48 h time point was chosen because it coincides with the first necrotic lesions in wildtype, but before the onset of widespread cell death. As shown in Fig. 5, ethylene insensitivity at ERS2 inhibits the expression of *ETR1* (2 fold), while *ERS2*, *ETR1* and *ETR2* (~3.3 fold each) were significantly repressed in the *etr2-1* mutant. The most striking differences in induction of receptor expression were observed in the *ers1-1*, *etr1-1* and *ein4-1* mutant backgrounds. In *ers1-1*, there were significant increases in *ERS2* (10 fold), *ETR1* (10 fold) and *ETR2* (7 fold) expression, while in *etr1-1* there was a large increase in *ERS1* (29 fold), *ERS2* (10 fold), *ETR1* (14 fold) and *EIN4* (11 fold) ($p < 0.05$). The largest change in expression to a single receptor was observed in *ein4-1*, with *ETR1* expression showing a 428 fold induction over 0 h levels. A more modest, but significant, increase in *ERS2* (3.5 fold) was also observed in this mutant ($p < 0.05$). These results suggest that signalling from ETR1 and ERS1 receptors has a role in negatively controlling the expression of other ethylene receptors during cell death, while signalling from ETR2 plays a role in the positive control of receptor expression.

4. Discussion

4.1. ETR1 and EIN4 have different roles in pathogen response

It has previously been shown that basal resistance to necrotrophic fungi (like *F. verticillioides*) requires an active ethylene signalling pathway [1,46–48]. In other instances, such as *Pseudomonas syringae* infection, insensitivity to ethylene does not confer pathogen resistance [49]. To follow the effect that loss of ethylene signalling has on the progression of cell death, most previous studies



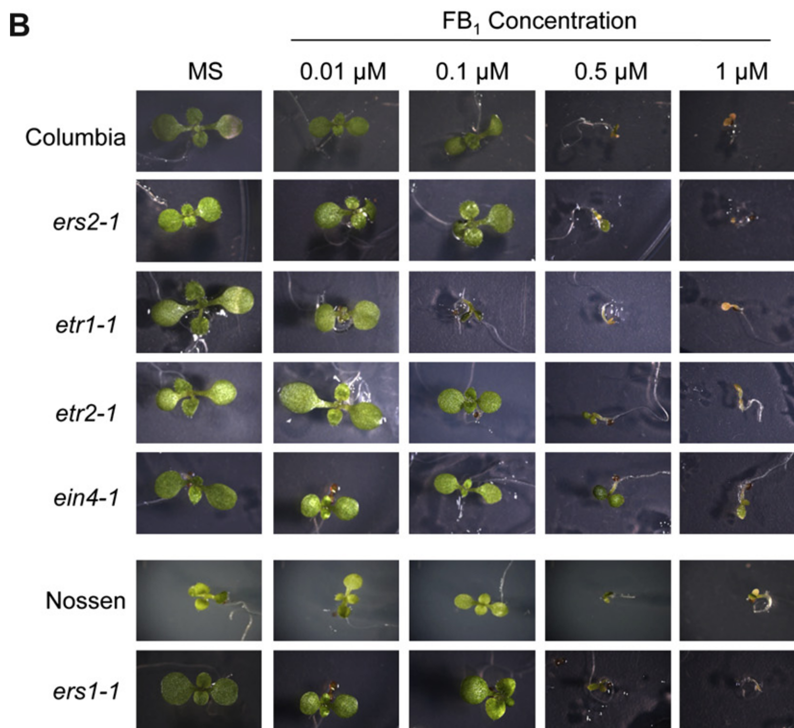


Fig. 1. Ethylene receptor mutants display differential rate of fumonisin B_1-induced cell death. (a) Representative images of leaves infiltered with 5 μM FB_1 and harvested every 12 h. Red underline indicates when the first visible signs of necrosis appeared in 50% of infiltered plants. Control leaves were infiltered with 0.14% methanol and were also harvested every 12 h. (b) Seeds of wildtype *Arabidopsis* and 5 gain-of-function ethylene receptor mutants were germinated on increasing concentrations of FB_1 to determine if any of the mutants were more resistant to FB_1. Representative images of each test were taken to indicate the extent of plant necrosis during this time period and to catalogue any death.

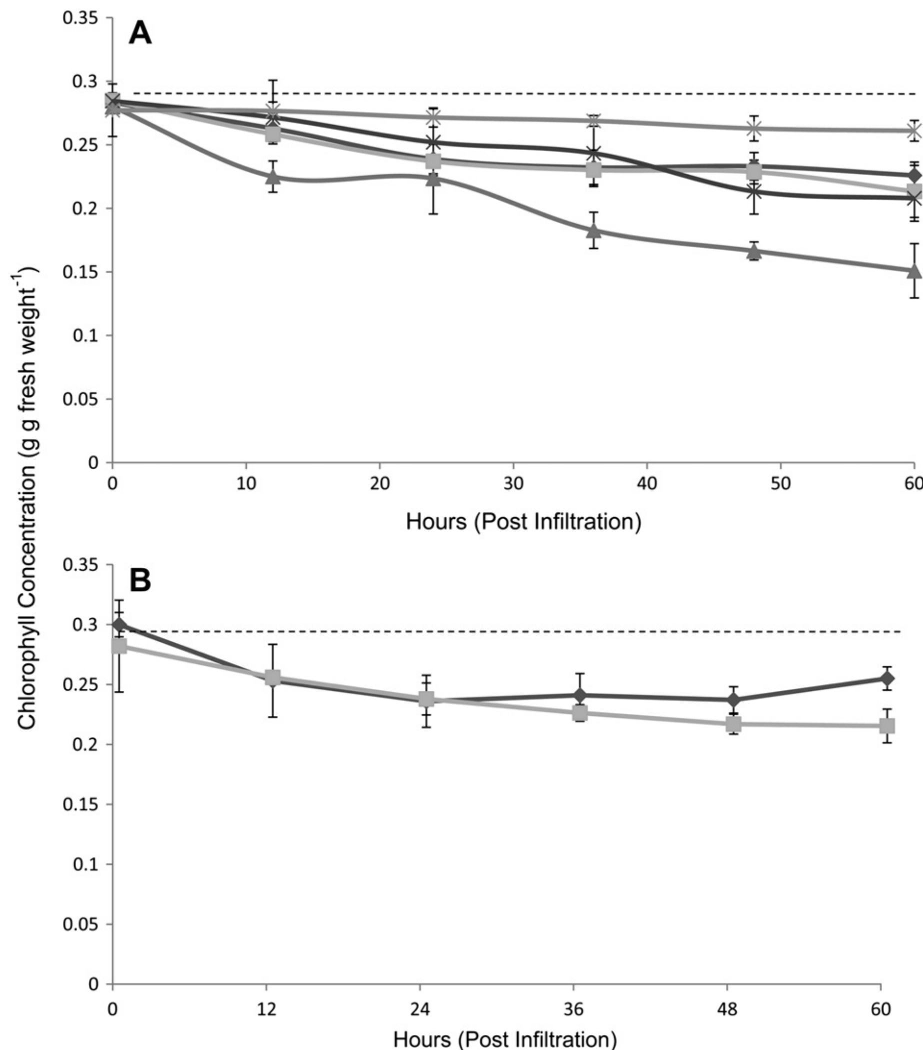


Fig. 2. Ethylene receptor mutants lose chlorophyll at different rates during fumonisin B_1-induced cell death. (a) Leaves of Columbia wildtype (light grey box), *etr1-1* (light grey triangle), *etr2-1* (dark grey x), *ers2-1* (dark grey diamond) and *ein4-1* (light grey x) were infiltered with FB_1 and total chlorophyll content in leaf discs was analyzed every 12 h. The dashed line indicates chlorophyll content in control leaves treated with 0.14% methanol across the time-course. (b) Chlorophyll content in Nossen wildtype leaves (dark grey diamond) and *ers1-1* (light grey box), sampled and treated as in (a). Error ± SE.

have focused on the *Arabidopsis etr1-1* gain-of-function mutant. It has been found that the *etr1-1* mutant has increased rates of cell death and reduced expression of defence genes such as *PDF1.2* [1,46]. New findings suggest that ETR1, and potentially ERS1, can act through signalling pathways other than the classical CTR1 dependent pathway [12,21,31]. This indicates that ETR1 is not representative of all the receptors. Therefore it is important to study the impact of the other 4 ethylene receptors on pathogen responses. Using the method developed by Stone and colleagues [1], we tested all five gain-of-function ethylene receptor mutants in *Arabidopsis* (*ers1-1*, *ers2-1*, *etr1-1*, *etr2-1* and *ein4-1*) for rates of mycotoxin-induced cell death (Fig. 1a). In contrast to *etr1-1*, *ein4-1* plants displayed delayed mycotoxin-induced cell death, as determined by chlorosis of FB_1 infiltered plants, while the other receptor mutants responded in a manner similar to wildtype. These results suggest that the ethylene receptors do not have redundant roles in pathogen response and, more specifically, that ethylene induced signalling from ETR1 would inhibit cell death while signalling from EIN4 in the presence of ethylene would accelerate cell death.

A potential explanation for the lack of cell death in *ein4-1* could be that FB_1 does not elicit HR-like induced lesions in this background due to a lack of callose and phenolic accumulation. FB_1 is known to induce an HR-like response during the formation of necrotic lesions in leaves marked by the deposition of callose and the accumulation of phenolics [1]. While there appeared to be a lower accumulation of callose and phenolics in *ein4-1* as compared to wildtype, which could lead to a delay in cell death, the variability between samples did not allow for accurate quantification of this difference. Our results, however, do demonstrate that callose and phenolics were induced by FB_1 administration in *ein4-1*, indicating that the absence of lesions in this mutant due to a repressed hypersensitive-like response is unlikely.

4.2. Ethylene receptors show differential control of defence pathways

The rate of cell death in response to pathogen attack has been shown to be influenced by a complex web of crosstalk between the ethylene, SA and JA pathways (for review see [4]). We wanted to extend previous work and address whether mutations to ethylene receptors other than ETR1 would have an impact on the expression defence genes. Here we have shown that *etr1-1* is not

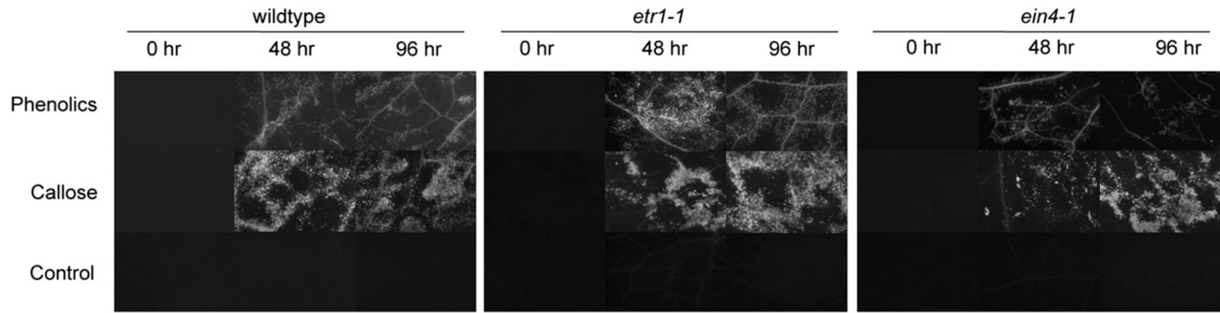


Fig. 3. Phenolic and callose accumulation in wildtype, *etr1-1* and *ein4-1*. Phenolic and callose staining in leaf discs of wildtype and the ethylene receptor mutants at 0, 48 and 96 h post-inoculation with FB_1. Control leaves were infiltered with an equivalent amount of 0.14% methanol.

representative of all ethylene receptors. Previous research had revealed that ethylene insensitivity can either induce JA specific genes [50] or leave expression levels unaffected [10,51]. *VSP1* is a gene involved in mediating JA's wound and pathogen response pathways [10]. We have shown that ethylene insensitivity at the ERS1 and ERS2 receptors leads to *VSP1* expression in the presence of FB_1. Absence of ethylene perception at ETR1, ETR2 and EIN4, meanwhile, did not affect the induction of *VSP1* during FB_1 treatment. These findings may explain the seemingly conflicting results of Lorenzo et al. [49] and Glazebrook et al. [50] by showing that

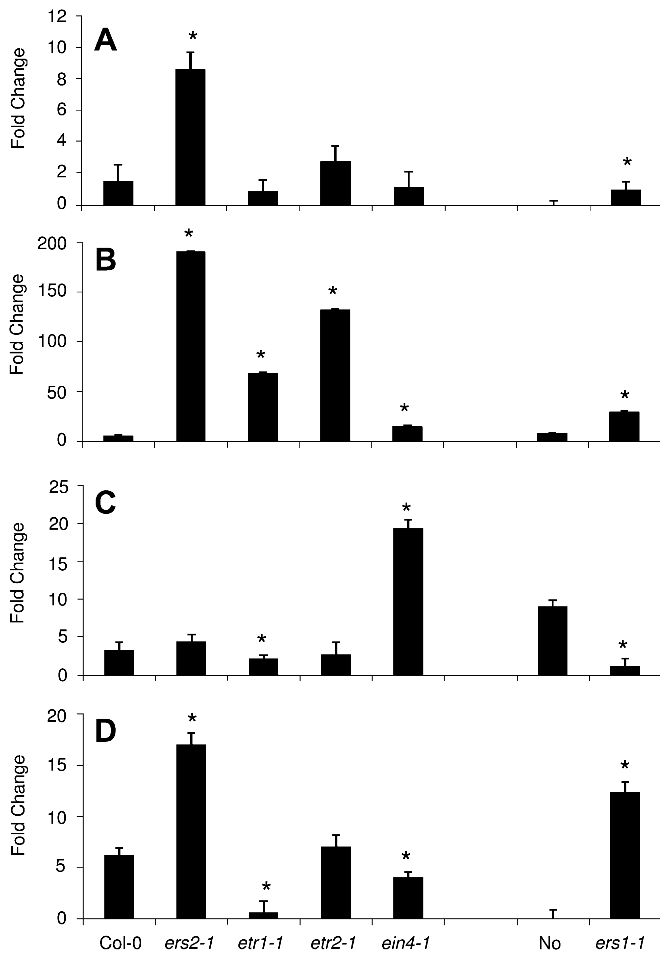


Fig. 4. Ethylene receptors mutants have differentially regulated defence genes in *Arabidopsis*. The seeds of wildtype *Arabidopsis* (Col-0 and No-0) and 5 gain-of-function ethylene receptor mutants were grown on 0.1 μM FB_1 for 10 days after which expression levels of (a) *VEGETATIVE STORAGE PROTEIN1 (VSP1)*, (b) *PATHOGENESIS RELATED1 (PR1)*, (c) *ETHYLENE RESPONSE FACTOR1 (ERF1)* and (d) *PLANT DEFENSIN1.2 (PDF1.2)* were analyzed. Values are expressed as fold change from each respective mutant grown on MS. Asterisk indicates significantly different induction or repression as compared to wildtype values ($p < 0.05$; *T*-test). Error ± SE.

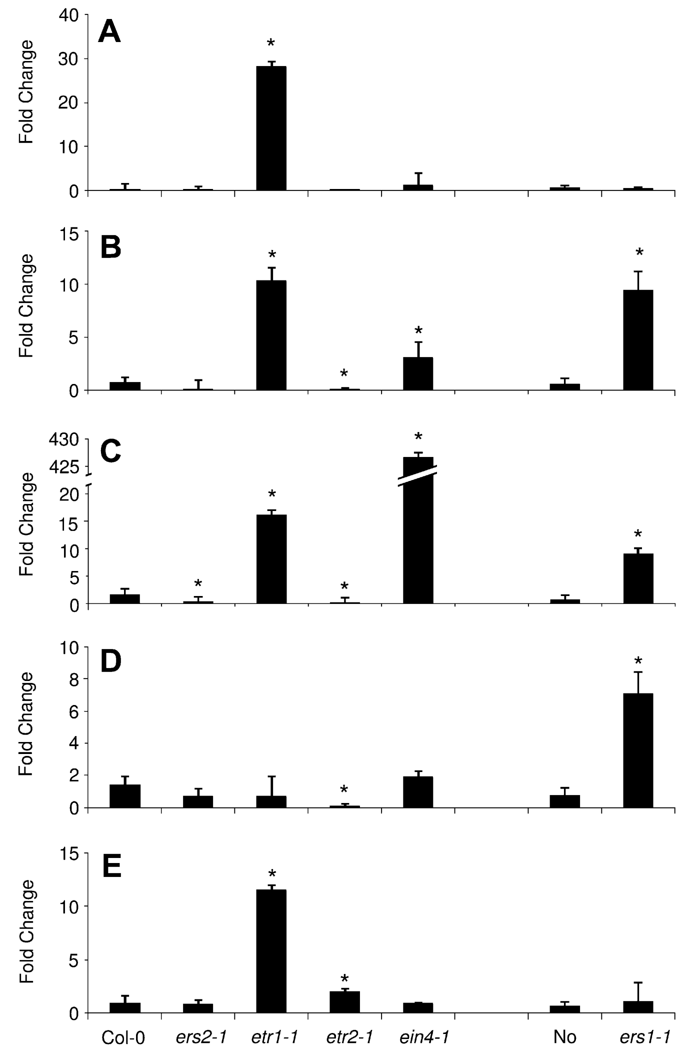


Fig. 5. Ethylene signalling plays a major role in regulation of ethylene receptors expression during fumonisin B_1 challenge. Expression levels of (a) *ERS1*, (b) *ERS2*, (c) *ETR1*, (d) *ETR2*, and (e) *EIN4* in wildtype (Col-0 and No-0) and 5 ethylene gain-of-function receptor mutants at 48 h after infiltration with 5 μM FB_1. Values are expressed as fold change from 0 h inoculation levels. Asterisk indicates significant difference from wildtype levels ($p < 0.05$). Error ± SE.

ethylene's effect on *VSP1* expression depends on which receptor binds ethylene.

The *PR1* gene is dependent on SA signalling for its expression and is used widely as a representative gene when analyzing induction of the SA pathway [1–3]. Ethylene represses *PR1* expression [52], therefore gain-of-function mutants of the ethylene receptors would be expected to increase the expression of SA inducible genes. This was observed in all five ethylene receptor mutants tested, though the degree to which the receptors are involved in repression of this pathway vary in their intensity. This may allow for fine-tuning of the ethylene response during FB_1-induced cell death.

Expression of genes such as *PDF1.2* also requires the coordinated signalling of the JA and ethylene pathways [4–6]. Experiments in JA-deficient (*coi1*) and ethylene insensitive (*etr1-1*, *ein2-1*) mutants showed reduced levels of *PDF1.2* [53]. In our results, however, we show that *PDF1.2* levels in *etr1-1* an *ein4-1* were decreased, while in *ers1-1* and *ers2-1* mutant backgrounds expression of *PDF1.2* was increased. These results suggest, contrary to the model based on *etr1-1* [53], that ethylene bound to ethylene receptors may induce or repress *PDF1.2*. These data would also indicate that it is possible for ETR1 and ERS1 receptors, two receptors that bear very close homology, to act antagonistically in the same pathway.

ERF1 is a transcription factor from the APETALA/ethylene-responsive-element binding protein family that is controlled by the activity of EIN3 [5,54]. As an ethylene responsive element, it requires the presence of an active ethylene signalling pathway to be expressed, and is expected to be inhibited in gain-of-function ethylene receptor mutants. This was shown to be true only in the ETR1 and ERS1 receptor mutants. In *etr2-1* and *ers2-1* the expression of *ERF1* was induced by FB_1 in a similar manner to wildtype, which would suggest that ETR2 and ERS2 have no role in the induction of *ERF1*. Of note, however, is that *ein4-1* has a much higher level of *ERF1* expression as compared to wildtype. This is completely contrary to the model developed using *etr1-1* as a representative model for the function of all ethylene receptors. This would indicate that signalling through EIN4 is normally responsible for the repression of *ERF1* expression. The increase in *ERF1* expression in *ein4-1* may also explain the heightened resistance of *ein4-1* to mycotoxins. There is a large body of evidence which indicates that overexpression of *ERF1* alone can induce resistance to necrotrophic pathogens [2,5,49]. Therefore, the high level of *ERF1* transcript accumulation may be responsible for the resistance of *ein4-1* to FB_1 observed.

Together these results suggest that the ethylene receptors have very different roles in hormone crosstalk and induction of defence signalling genes as compared to the model put forth based on the response of *etr1-1*. In light of cell death phenotypes identified in the receptor mutants, *etr1-1* likely has a fast cell-death response due to the repressed defence signalling pathways while *ein4-1* resistance to FB_1 may be due to overexpression of ERF1.

4.3. Ethylene insensitivity alters ethylene receptor transcript accumulation

A complicating factor in understanding the role of each receptor is the possibility that mutations in any one receptor could impact the level of expression of the other ethylene receptors. This feedback has been shown to occur in other studies [12,45,55]. Therefore, we evaluated whether a gain-of-function mutation in any one of the ethylene receptors could influence the expression of the other receptors. It was found that no one receptor was highly up-regulated or repressed during FB_1-induced cell death in wildtype. In the ethylene receptor mutants it was found that *ers1-1* had increased expression of *ERS2*, *ETR1* and *ETR2*; *etr1-1* had increased expression of *ERS1*, *ERS2*, *ETR1* and *EIN4* and *ein4-1* had increased expression of *ETR1* and *ERS2*. Conversely, *etr2-1* had repressed expression of *ERS2*, *ETR1*, *ETR2* and *EIN4*. These results would indicate a role for ERS1, EIN4 and ETR1 in a negative control of ethylene receptor expression while ETR2 plays an important role in the positive transcriptional control of several ethylene receptors. It is also interesting to note that in the *ein4-1* background, *ETR1* expression is increased substantially while in the *etr1-1* background we observed a significant, and substantial, increase in *EIN4* transcription. Given our findings that ETR1 and EIN4 have opposing roles in cell death, this may indicate that in *etr1-1* an increase in the level of EIN4 may initiate or coordinates signalling that leads to cell death, while in *ein4-1* an increase in ETR1 inhibits signalling relays which would normally lead to cell death. As ETR1 is responsible for induction of *ERF1* upon binding of ethylene, the large increase in *ERF1* expression in *ein4-1* may be the result of signalling from ETR1. Conversely, repression of *ERF1* in *etr1-1* may be due to signalling from EIN4. This may indicate that loss of ethylene sensitivity at any one receptor may not fully mask the signalling from other receptors during certain stages of plant development as first thought [22–26]. Further research is needed to determine the effect that upregulation of the other receptors have in GOF backgrounds.

From FB_1-induced cell death, the evidence points to major roles for EIN4 and ETR1, where ethylene signalling through EIN4 induces cell death and ethylene signalling through ETR1 inhibits cell death. We have proposed a model that summarizes the individual roles of the ethylene receptors in signalling cell death in the presence of ethylene, with specific emphasis on the role of ETR1 and EIN4 (Fig. 6a). Ethylene signalling from ETR1 and EIN4 regulates the expression of other ethylene receptor genes (Fig. 6b). Together the 5 ethylene receptors co-ordinately impact the expression of downstream defence genes (Fig. 6c). Our results suggest that when ethylene binds at EIN4, cell death is accelerated, potentially through repression of *ERF1*, a gene known to be essential for pathogen resistance. ETR1 has an opposite effect on the rate of cell death, most likely through positive regulation of *PDF1.2* and *ERF1* when ethylene is present. The finding that ETR1 and EIN4 are major players in the control of each others expression opens up the possibility that the balance of expression between the two receptors affects, or decides,

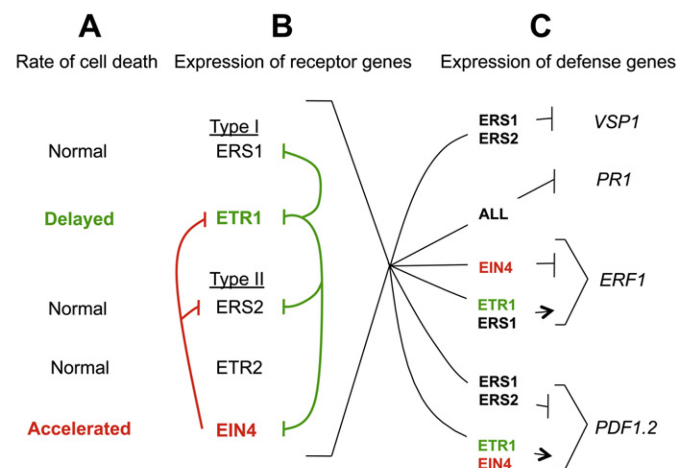


Fig. 6. Proposed model of the involvement of the 5 *Arabidopsis* ethylene receptors in mediating mycotoxin-induced cell death. Application of the mycotoxin FB_1 induces the production of ethylene which can bind to the 5 ethylene receptors located in the endoplasmic reticulum. (a) Rate of cell death as compared to wildtype. Our data suggests that binding of ethylene to EIN4 (color-coded in red) would accelerate cell death, while binding of ethylene to ETR1 (color-coded in green) would delay cell death. (b) When ethylene binds to ETR1, it leads to an inhibition in the expression of all receptors, including itself, except ETR2. Ethylene bound at EIN4 represses ETR1 and ERS2 expression. (c) All of the ethylene receptors co-ordinately signal to control downstream defence genes both in the ethylene mediated defence pathway (*ERF1* and *PDF1.2*) and/or in the JA pathway (*VSP1*) and in the SA pathway (*PR1*). Arrows indicate positive control while flat arrow indicates repression.

the outcome of cell death in response to mycotoxins. This study gives a framework that helps explain the contradictory effect of ethylene on cell death and pathogen resistance as each ethylene receptor in *Arabidopsis* has non-redundant roles during defence pathway activation and mycotoxin-induced cell death.

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