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Resistance of *Fusarium poae* in *Arabidopsis* leaves requires mainly functional JA and ET signaling pathways

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ABSTRACT

Fusarium poae has been considered as a minor species among those that cause the FHB disease but in recent years several researchers have documented a high frequency of occurrence in several crops. We evaluated the ability of *F. poae* to produce symptoms in *A. thaliana* leaves. Moreover, we analyzed the defense of *A. thaliana* against *F. poae* using SA, JA, and ET mutants and we monitored the expression level of genes involved in the main signaling pathways related to plant defense. Symptoms were observed in the inoculated leaves demonstrating the ability of *F. poae* to infect *A. thaliana* leaves. Moreover, the *npr1-1* mutants presented low symptoms compared to Col-0, *etr2-1*, and *coi1-1* and that the *coi1-1* mutant was the most susceptible genotypes followed by *etr2-1* genotypes. The RT-PCR revealed that *PDF1.2*, *CHI/PR3*, and *ERF1*, three important JA-ET responsive genes and *NPR1* and *PR1*, which are regulated by SA signaling, were expressed upon *F. poae* inoculation. Our results suggest that JA and ET could play a key role in *Arabidopsis* leaves defense against *F. poae* representing the first evaluation of the response of the main *A. thaliana* phytohormones involved in plant defense in the presence of *F. poae*.

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Introduction

In interaction with the environment, plants are often exposed to different types of stress including abiotic stress caused by temperature or water availability, and biotic stress such as diseases caused mainly by viruses, bacteria or fungi. Disease represents a major cause of the negative impacts of biotic stress on crop yields. One of the most important fungal diseases of small grain cereals is Fusarium Head Blight (FHB) by reducing barley, wheat and oat production and seed quality because of the ability of *Fusarium* species to produce mycotoxins harmful to both human and animal health (McMullen

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et al. 1997; Desjardins 2006). Among *Fusarium* species, *Fusarium graminearum*, *Fusarium poae*, *Fusarium avenaceum*, and *Fusarium culmorum* have been frequently isolated from plant tissue exhibiting FHB symptoms whose occurrence depend on the environment conditions in the moment that disease develops (Nicholson et al. 2003). Commonly, *F. poae* is considered as a minor species due to be less pathogenic and aggressive than other FHB pathogens as *F. graminearum*, but in the last years several researchers have documented a high frequency of occurrence worldwide (Audenaert et al. 2009; Stenglein et al. 2012; Lindblad et al. 2013). Infantino et al. (2012) demonstrated that *F. poae* was the most dominant species isolated from Italian wheat. Recently, Nielsen et al. (2014) showed that *F. poae* is the prevalent species in barley grain in the United Kingdom affecting the quality and safety of malt and beer.

Fusarium poae is a necrotrophic pathogen able to produce several mycotoxins not only type A such as HT-2, T-2 and diacetoxyscirpenol (DAS) and B trichothecenes such as nivalenol (NIV) with harmful effects on human and animal health but also other minor mycotoxins such as enniatins, beauvericin, neosolaniol with minor effects on consumers but equally important (Gutleb et al. 2002; Thrane et al. 2004; Meca et al. 2010). Moreover, *F. poae* is considered the most important NIV producer on barley, wheat, maize, and oats (Vogelgsang et al. 2008).

Plants produce several hormones essential for the regulation of plant growth, development, reproduction, and survival. Phytohormones include auxins (AUX), gibberellins (GA), abscisic acid (ABA), cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonates (JA), brassinosteroids (BR), and peptide hormones, which change their levels during pathogen infection (Adie et al. 2007; Bari & Jones 2009). The induced defense responses are regulated by a network of interconnecting signal transduction pathways in which SA, JA, and ET play key roles. Therefore, the plant resistance to biotrophic pathogens is thought to be mediated through SA signaling, while resistance to necrotrophic pathogens is mediated by JA/ET (Glazebrook 2005).

Several plants have been used as model system to study the plant–pathogen interaction. The most recognized system is the crucifer *Arabidopsis thaliana* L. which has several characteristics that facilitate *Arabidopsis* genome manipulation providing different signaling pathway mutants and transgenic lines useful for plant–pathogen interaction studies (Dangl 1993). Several authors have evaluated the interaction between *Fusarium* species and *Arabidopsis*. Chen et al. (2006) and Makandar et al. (2010) evaluated the behavior of *F. graminearum* on different ecotypes and several mutants of *Arabidopsis* in the main signaling pathways associated with plant defense, respectively. Moreover, Pantelides et al. (2013) used this model to study the *Fusarium oxysporum* pathogenicity. To our knowledge, no previous studies have evaluated the interaction between *F. poae* and *Arabidopsis*. Such studies would provide valuable information regarding the signaling pathways involved in plant defense against *F. poae*. Therefore, the objectives of this study were 1) to test the ability of *F. poae* to infect and to produce symptoms in *A. thaliana* leaves by using two different methods, 2) to evaluate the role of SA, JA, and ET role in the plant defense by testing pathogen virulence on mutant plants deficient in signaling pathways and evaluating expression levels of several genes involved in plant defense.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) as wild type (WT) and *npr1-1* [CS3796], *coi1-1* [CS68796], and *etr2-1* [CS67924] mutants were used. All the mutants used were in Col-0 background. Seeds were surface sterilized with 50 % ethanol for 3 min, then in 2 % sodium hypochlorite for 3 min and finally rinsed three times in sterile distilled water. All seeds were vernalized at 4 °C and were sown into 8-cm-diameter pots, each containing approximately 200 cm^3 of sterilized soil: perlite: vermiculite mixture (4:1:1) at 20 °C–24 °C with 16 h of light (150 $\mu E\ m^{-2}\ s^{-1}$) in a controlled environmental growth chamber. The plants were watered as needed.

Fungal isolates and inoculum preparation

A total of four *Fusarium poae* isolates were selected based on high level of nivalenol production according to the *in vitro* nivalenol production evaluated by Dinolfo et al. (2012) and were conserved on Spezieller Nährstoffarmer Agar (SNA) slants according to Leslie & Summerell (2006). Before being used for inoculation, fungal isolates were cultured in Petri dishes containing potato dextrose agar (PDA) at 25 °C under 12 h light/dark conditions for 5–7 days. Conidial harvest was taken by flooding the plates with 5 ml of distilled water and dislodging the conidia with a bent glass rod. The resulting suspension of the mixture of the four isolates selected was filtered through cheesecloth and the conidial suspension was adjusted to 1×10^5 conidia per ml according to Brennan et al. (2007) using a haemacytometer (Neubauer) and a binocular microscope. Tween® 20 (0.05 %) (Biopack) was added as surfactants.

Fusarium poae – Arabidopsis thaliana *assays*

Four week old plants were used for inoculation. Two methods were used to assess virulence. First, the adaxial surfaces of the leaves were wounded and the inoculum was deposited on the wound site as described by Chen et al. (2006). Second, the conidial suspension was infiltrated into the *A. thaliana* abaxial leaf surfaces with a syringe according to Makandar et al. (2010). In both cases, control plants were inoculated with sterile distilled water plus Tween® 20 (0.05 %) (Biopack) and all the plants were covered with polythene bags to maintain high relative humidity. A total of three leaves per plant were inoculated with *F. poae*. The experiment was repeated three times with 20 replicates per experiment. Disease severity (DS) was evaluated by a disease score according to Chen et al. (2006) and was daily recorded for 30 days after inoculation to generate the disease progress curve. The DS index was scored visually and the rating used was: 0, no symptoms; 1, chlorotic lesion restricted to the inoculation site; 2, chlorotic lesion covering 25 of the leaf area; 3, chlorotic lesion covering 25–50 % of the leaf area; 4, chlorotic lesion covering 50–75 % of the leaf area; 5, chlorotic lesion covering the entire leaf (Chen et al. 2006). The area under the disease curves (AUDPC) was calculated by the trapezoidal integration method (Campbell &

Madden 1990) and the disease was expressed as a percentage of the maximum possible area for the whole period of the experiment known as relative AUDPC. ANOVA analyses were performed and the levels of significance were established by using Tukey's test at $p \leq 0.05$.

Nucleic acids isolation and RT-PCR analysis

Regarding DNA and RNA isolation, leaf samples were collected at 24, 48, and 72 h post-inoculation (hpi) in liquid nitrogen. Total genomic DNA from leaves was extracted using the cetyltrimethylammonium bromide (CTAB) method according to Stenglein & Balatti (2006) and the resulting DNA was examined by electrophoresis in 0.8 % (w/v) agarose gels containing GelRed™ (Biotium; Hayward) at 80 V in 1X Trisborate-EDTA buffer for 3 h at room temperature. The DNA visualization was made under UV light and the DNA concentration was calculated using a fluorometer (Qubit Fluorometer, Invitrogen). As regards Koch's postulates, a species-specific PCR was used to test the *Fusarium poae* presence in the inoculated and control leaves according to Parry & Nicholson (1996) using a *F. poae* isolate (Hsu1a) as a positive control and a *Fusarium sporotrichioides* isolate (F95) as a negative control previously described in Dinolfo *et al.* (2010). For RNA extraction, TRI® reagent (Sigma) was used following the manufacturer's instructions. The dry pellet was dissolved in 20 μl of distilled water and was stored at −80 °C. Prior to cDNA synthesis, the RNA concentration was calculated using a fluorometer (Qubit Fluorometer; Invitrogen). Total RNA (1 μg) was used to synthesize cDNA using M-MuLV reverse transcriptase (New England Biolabs, Hitchin, Hertfordshire, UK) in a final volume of 30 μl. The cDNA was used as template. RT-PCRs using CHI/PR3, PDF1.2 and ERF1 primers were used to observe the expression of genes linked with ET/JA signaling pathways as *CHI/PR3* (*CHITINASE/PATHOGENESIS-RELATED 3*), *PDF1.2* (*PLANT DEFENSIN 1.2*), and *ERF1* (*ETHYLENE-RESPONSE FACTOR 1*), respectively. Conversely, NPR1 and PR1 primers were performed to evaluate the expression of *NPR1* (*NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1*) and *PR1* (*PATHOGENESIS-RELATED 1*), respectively, as the common markers for SA-related defense genes. The expression of these genes was normalized with *Actin* gene expression. Primers used for RT-PCR reactions are listed in Table 1. The PCR products were separated in 1 % agarose gels and the fragment intensity was quantified using TotalLab v1.10 demo software. The RT-PCRs were repeated three times. The relative mRNA level was calculated with respect to the level of the corresponding transcript in control plants. ANOVA analyses were performed and the levels of significance were established by using Tukey's test at $p \leq 0.05$.

Results

Fusarium poae *symptoms*

Only one of the inoculation methods produced disease symptoms. The deposited *F. poae* inoculum on wounded leaves showed no visible symptoms in any of the leaves inoculated during the 30 days post-inoculation (dpi). Conversely, the leaf infiltration method described by Makandar *et al.* (2010) showed visible symptoms at 6 dpi and the symptoms were recorded until 30 dpi. DS progressed rapidly in all genotypes, except for *npr1-1* which showed less symptoms and comparatively less disease development (Figs 1–2A). At 30 dpi, the DS of Col-0 was 64 % while *coi1-1* mutants showed 85 % the highest percentage of DS relative to WT. Regarding *etr2-1*, the DS was an average of 79 % and the *npr1-1* mutants showed the lowest percentage (30 %) (Fig 2A). The ANOVA revealed that the average AUDPC for *coi1-1* was significantly more diseased than the other genotypes. Conversely, the *npr1-1* mutants exhibited less visible symptoms compared to the other ones ($p \leq 0.0001$) (Fig 2B).

All DNA samples from *F. poae* inoculated leaves amplified the expected 220 bp fragment as previously observed by Parry & Nicholson (1996) while no amplification was observed in DNA from control leaves (Fig S1) suggesting that the symptoms were indeed caused by *F. poae*.

Defense responses triggered by Fusarium poae

Regarding the expression of genes linked with ET/JA signaling pathways, *PDF1.2*, *CHI/PR3*, and *ERF1* were analyzed. On the other hand, the expressions of *NPR1* and *PR1* as SA-related defense genes were evaluated (Fig 3). In WT plants, the treated/mock plants ratio showed that *CHI/PR3*, *ERF1*, *NPR1*, and *PR1* expression increased at 24 hpi, decreased at 48 hpi and increased again at 72 hpi while the *PDF1.2* expression increased with time. The genotype with less visible symptoms, *npr1-1*, showed the highest *PDF1.2*, *CHI/PR3*, *ERF1*, *NPR1* expression at 24 hpi, decreasing with the time while the *PR1* expression increased with the time. The most susceptible genotype, *coi1-1*, showed the *CHI/PR3*, *ERF1*, *NPR1*, and *PR1* expression similar to WT increasing at 24 hpi, decreasing at 48 hpi and finally increasing again at 72 hpi. Unlike WT, the *PDF1.2* expression increased at 24 hpi, decreased at 48 hpi and increased again at 48 hpi. Generally, *coi1-1* showed more expression in *PDF1.2*, *CHI/PR3*, *ERF1*, and *NPR1* compared to WT. Regarding *etr2-1* genotypes, the expression of the genes evaluated were different not only compared to WT but also to *npr1-1* and *coi1-1*. The *PDF1.2* and *NPR1* expressions were higher at 48 hpi compared to 24 hpi and 72 hpi. The *CHI/PR3* expression was higher at 24 hpi but no differences in gene expression between treated and mock plants were observed at 48 hpi and 72 hpi. The *ERF1* expression increased at 24 hpi decreasing with time. As regard *PR1*, gene expression was observed at 48 hpi increasing with time. In conclusion, the most noticeable difference between genotypes was not the expression of genes involved but the time and the level in expression in which genes were expressed after infection.

Discussion

Fusarium poae is becoming a more prevalent causal agent of FHB which reduces crop yields and causes food safety concerns. However, not much is known about the defense response of plants to *F. poae*. This study represents the first

Table 1 – Primer sequences used for RT-PCR reactions.

Species	Primers	Sequence (5′–3′)	Reference
Arabidopsis thaliana	PDF1.2F	CTG TTA CGT CCC ATG TTA AAT CTA CC	Pantelides et al. (2013)
	PDF1.2R	CAA CGG GAA AAT AAA CAT TAA AAC AG	
	NPR1F	GTC TTC TCC GCA AGC CAG TTG A	Pantelides et al. (2013)
	NPR1R	AAC CGT GGA ACT CGG GAA ACG A	
	PR1F	TCA CAA CCA GGC ACG AGG AG	Pantelides et al. (2013)
	PR1R	CAC CGC TAC CCC AGG CTA AG	
	CHI/PR3F	TTA TCA CCG CTG CAA AGT CCT	Pantelides et al. (2013)
	CHI/PR3R	TGG CGC TCG GTT CAC AGT A	
	ERF1F	CCT TCC GAT CAA ATC CGT AAG	Camehl et al. (2010)
	ERF1R	TCC CGA GCC AAA CCC TAA TAC	
	Actin2/8F	GGT AAC ATT GTG CTC AGT GGT GG	Asano et al. (2008)
	Actin2/8R	AAC GAC CTT AAT CTT CAT GCT TGC	
Fusarium poae	FP82F	CAA GCA AAC AGG CTC TTC ACC	Parry & Nicholson (1996)
	FP82R	TGT TCC ACC TCA GTG ACA GGT T	

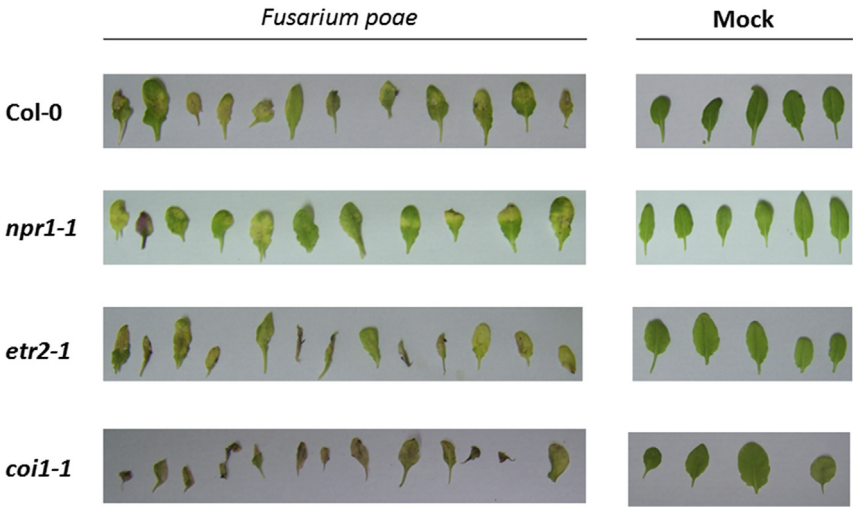

Fig 1 – Symptoms caused by *Fusarium poae* on *Arabidopsis* Col-0, *npr1-1*, *etr2-1*, and *coi1-1* mutants at 30 days post-inoculation.

evaluation of *Arabidopsis thaliana* phytohormones in the presence of *F. poae* by using WT and mutants of the SA, JA, and ET signaling pathways. Symptoms were observed in the inoculated leaves which allow demonstrating the ability of *F. poae* to infect and to develop in *Arabidopsis* leaves. Regarding other *Fusarium* species, Urban et al. (2002) demonstrated that both *Fusarium graminearum* and *Fusarium culmorum* infect *Arabidopsis* floral and silique tissue. Moreover, *Fusarium oxysporum* was another *Fusarium* species widely evaluated in *Arabidopsis* showing symptoms in the model used (Berrocal Lobo & Molina 2004). Later, Chen et al. (2006) used *F. graminearum* to inoculate not only intact *Arabidopsis* plants and florets but also leaves using a detached leaf assay and observed *Fusarium* symptoms in all treatments. In our study, the same method applied by Chen et al. (2006) was used to inoculate *Arabidopsis* leaves and no symptoms were observed in any of the leaves inoculated. Differences in the method applied to inoculate and the pathogen used in each study could explain the differences in the results found in both assays. Regarding the methods, the infiltration described by Makandar et al. (2010) ensures the pathogen enters the leaves while the method described by Chen et al. (2006) deposit the conidial suspension on the wound site of the leaves and being *F. poae* a weak pathogen compared to *F. graminearum*, the pathogen is not able to enter easily the leaves and for this reason no visible symptoms were observed in the time evaluated. On the other hand, *F. graminearum* is able to produce deoxynivalenol (DON), a type B trichothecene able to play a key role on the disease process modulating a cell-death pathway (PCD) and producing reactive oxygen species (ROS) as a strategy for plant colonization making *F. graminearum* a specialized pathogen instead of the more opportunistic *F. poae* (Chen et al. 2009). There are evidences that suggest that DON could contribute to *F. graminearum* virulence in wheat but it is not required for *F. graminearum* to infect *Arabidopsis* floral tissue (Cuzick et al. 2008a,b). Harris et al. (1999) and Proctor et al. (1995) demonstrated that trichothecene-producing isolates of *F. graminearum* were able to colonize

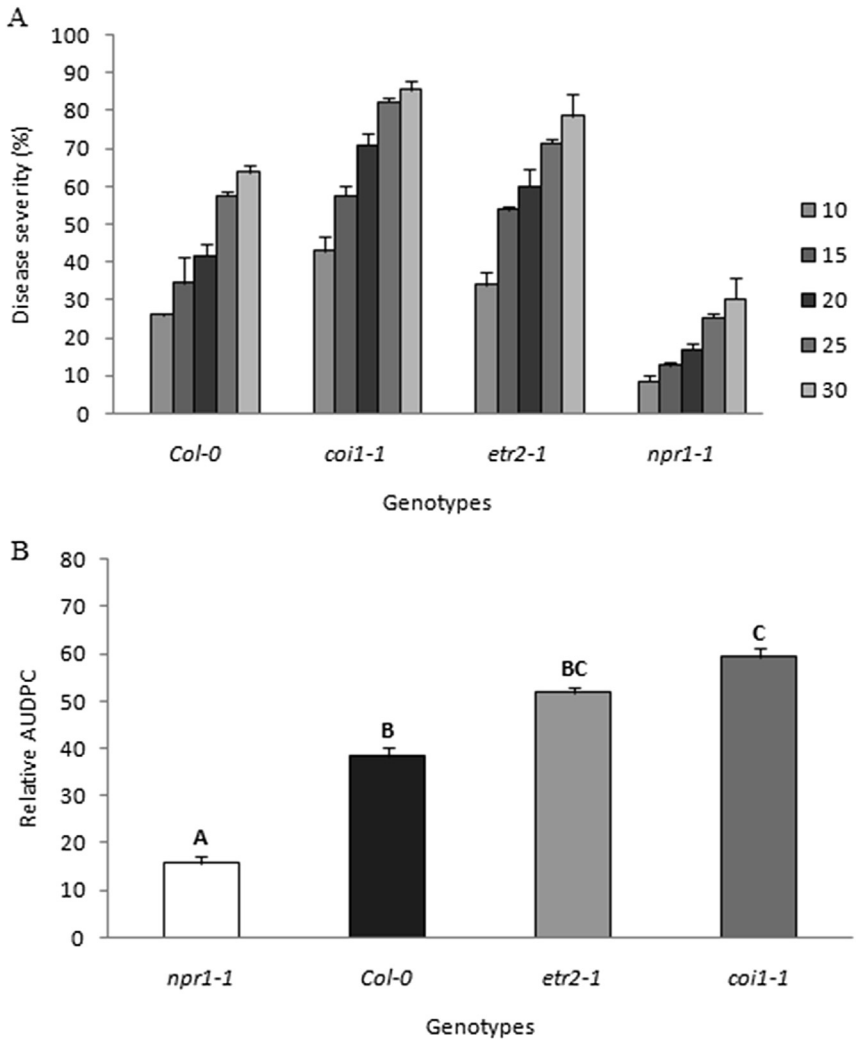

Fig 2 – (A) Percentage of disease severity of *Arabidopsis thaliana* after inoculation by *Fusarium poae*. A total of three leaves per plant were inoculated with *F. poae* and the symptoms were recorded at 10, 15, 20, 25, and 30 days post-inoculation. The experiment was repeated three times with 20 replicates per experiment. Columns represent means of 60 plants and the vertical bars indicate standard errors. (B) Relative AUDPC of the evaluated genotypes. The disease was expressed as a percentage of the maximum possible area for the whole period of the experiment represented by columns. The vertical bars indicate standard errors and the columns with different letters are statistically different according to Tukey's test at $p \leq 0.05$.

maize ears, wheat and winter rye more virulently than trichothecene-nonproducing isolates. Otherwise, *F. poae* is not able to produce DON hence the virulence is lower than *F. graminearum* so this could explain the absence of *F. poae* symptoms in the method described by Chen *et al.* (2006). Regarding the interaction with *F. poae*, the *npr1-1* genotype presented the lowest visible symptoms among the evaluated genotypes. NPR1 is an important signaling component in SA signaling and a key regulator of SA-mediated suppression of JA signaling. However, in *npr1-1* genotype the JA suppression is completely abolished enhancing JA response needed to defend the plant for necrotrophic pathogen attack (Spoel *et al.* 2003; León-Reyes *et al.*, 2009). In other studies using different *Fusarium* species, the *npr1-1* genotype increased the plant susceptibility to *Fusarium*. Cuzick *et al.* (2008a,b) evaluated the *npr1* mutant inoculated with *F. culmorum* and determined that the mutation of *NPR1* gene enhanced susceptibility to *F. culmorum* in *Arabidopsis* bud and flowers. Makandar *et al.* (2010) observed that *npr1* mutant results in heightened susceptibility to *F. graminearum* in *Arabidopsis* leaves and inflorescence. Moreover, mutants with constitutively SA signaling showed less visible symptoms compared to control plants suggesting that SA may play a key role in plant defense against *F. graminearum*. In our study, the *coi1-1* genotype showed more visible symptoms compared to the remaining genotypes evaluated. COI1, an important JA signaling component, has been shown to be necessary for resistance against pathogen with necrotrophic lifestyles; therefore a mutation in *coi1-1* increases the susceptibility to this kind of pathogen (Glazebrook 2005). Unlike our results, Thatcher *et al.* (2009) evaluated the interaction between *F. oxysporum* and *coi1-1* mutants and observed resistance to this pathogen when COI1 is not functional. Regarding the expression of several genes related to *F. poae* defense, *NPR1* gene

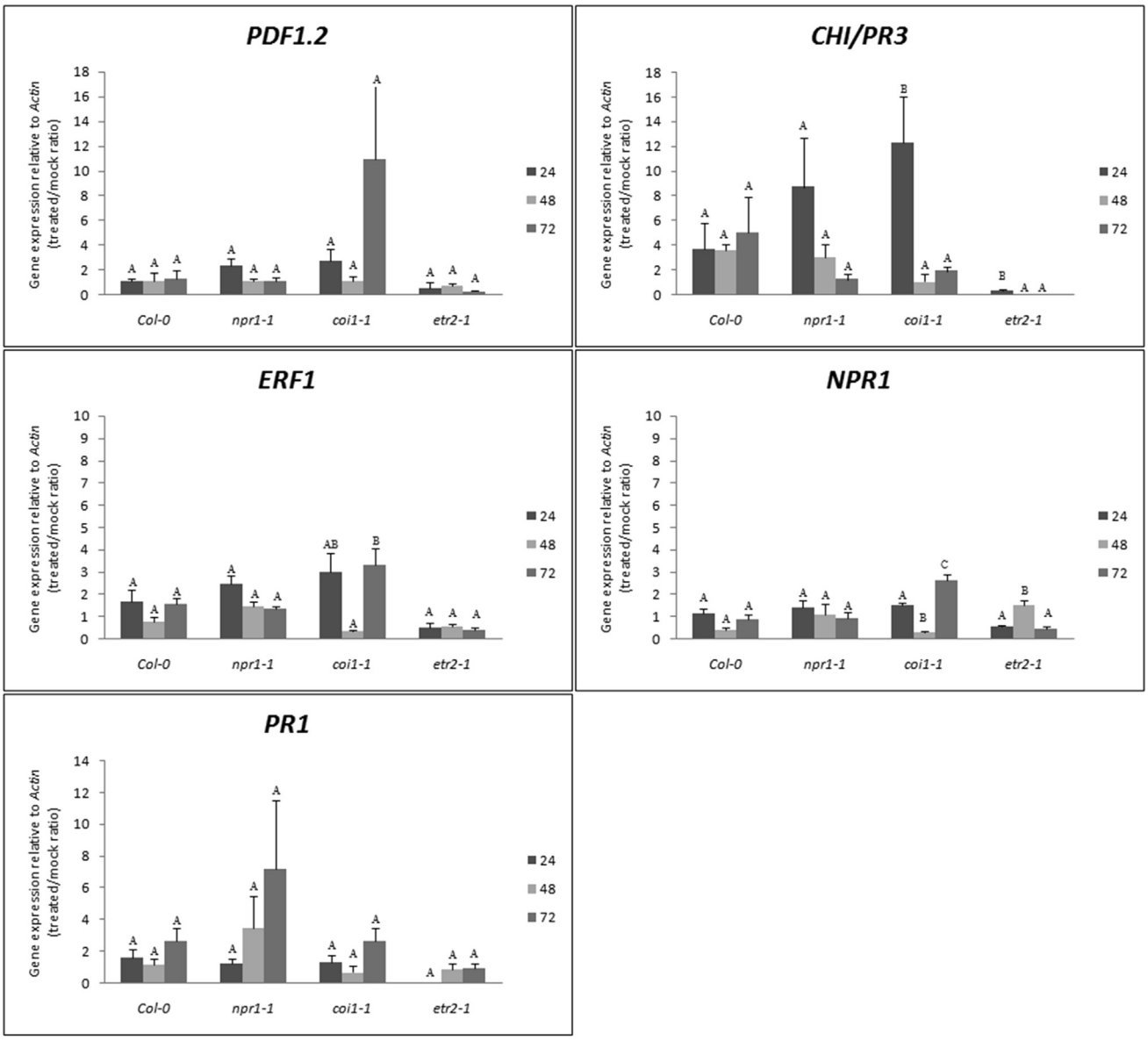

Fig 3 – Expression of PDF1.2, *CHI/PR3*, *ERF1*, NPR1, and PR1 in *Arabidopsis thaliana* Col-0, *npr1-1*, *coi1-1* and *etr2-1* plants in response to infection with *Fusarium poae* during 24, 48, and 72 h post inoculation (hpi). Expression levels were analyzed by RT-PCR and normalized against *Actin* gene. The relative mRNA level was calculated with respect to the level of the corresponding transcript in control plants. The columns represent the means of three technical replicates and the vertical bars indicate the standard errors. Different letters above the bars indicate significant differences according to Tukey's test at $p \leq 0.05$.

was induced in all the genotypes evaluated. Moreover, *PR1* gene, which is regulated by SA presence and is downstream NPR1, is also expressed in our study, indicating that monomeric NPR1 is traslocated to the nucleus where interacts to promote the *PR1* transcription as described by Mukhtar *et al.* (2009). Makandar *et al.* (2012) have demonstrated that *F. graminearum* has an initial activation of SA basal signaling during the infection and then, during the later stages of infection, promotes the JA signaling, showing a cross-talk between signaling pathways. Similar results were found by Asano *et al.* (2012) by using *Fusarium sporotrichioides* to inoculate *Arabidopsis* leaves. Regarding ET, the *etr2.1* genotype was more susceptible compared to WT; therefore ET signaling would be involved in the plant defense against *F. poae*. According to these observations, Chen *et al.* (2009) showed that *Arabidopsis* mutants with reduced ET perception were more susceptible to *F. graminearum* thus demonstrating that ET signaling is needed for *F. graminearum* resistance. Moreover, Pantelides *et al.* (2013) demonstrated that ETR1 is required for *F. oxysporum* pathogenicity because *etr1-1* plants showed less visible symptoms compared to control plants. *CHI/PR3* and *ERF1*, two downstream components of the ET-JA signaling pathways showed a higher level of expression upon *F. poae* infection. Berrocal Lobo *et al.* (2002, 2004) demonstrated that *ERF1* was expressed after *Botrytis cinerea* infection and that *ERF1* constitutive expression increased *Arabidopsis*

resistance to *B. cinerea*, *Plectosphaerella cucumerina*, and *F. oxysporum* thus showing that this regulator of ethylene responses confers resistance to several necrotrophic fungi. *PDF1.2* is an antifungal peptide induced by both JA and ET pathways. *PDF1.2* is a target of *ERF1*, hence, as expected, *PDF1.2* was expressed in all the genotypes (Solano *et al.* 1998). Although previous works observed that JA-gene expression is impaired in *coi1-1* mutants, our results show that *PDF1.2* is also induced in *coi1-1* mutants similar to those found by Thatcher *et al.* (2009). Wang *et al.* (2015) evaluated the plant defense against *Sclerotinia sclerotiorum*, another important necrotrophic fungal pathogen, by using *Arabidopsis* mutants in another JA/ET component known as Mediator complex subunit (MED16). Interestingly, *med16* reduced significantly the basal expression of *PDF1.2* compared to *coi1-1* demonstrating a key role of MED16 not only in the *PDF1.2* expression but also in the plant defense against necrotrophic pathogens.

Regarding *F. poae*, the *coi1-1* and *etr2-1* mutants of *Arabidopsis* exhibited statistically more symptoms compared to *npr1-1* mutant suggesting that JA-ET would play a key role in *Arabidopsis* defense against *F. poae* as expected for a necrotrophic pathogen. However, regarding the expression of defense genes, our results demonstrated that not only genes linked with JA-ET signaling but also SA related defense genes were expressed after the inoculation of *F. poae*. However, although SA responsive genes were expressed, the low symptoms observed in *npr1-1* mutants indicate that functional SA would not be required for plant defense to *F. poae*. In conclusion, our results represent the first evaluation of the response of the main *A. thaliana* phytohormones involved in plant defense in the presence of *F. poae*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2017.06.001.

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