

# *Botrytis cinerea* induces local hypoxia in *Arabidopsis* leaves

Maria Cristina Valeri<sup>1</sup> , Giacomo Novi<sup>1</sup> , Daan A. Weits<sup>1</sup> , Anna Mensuali<sup>1</sup> , Pierdomenico Perata<sup>1</sup>  and Elena Loreti<sup>2</sup> 

<sup>1</sup>PlantLab, Institute of Life Sciences, Scuola Superiore Sant'Anna, Via Giudiccionni 10, 56010 San Giuliano Terme (Pisa), Italy; <sup>2</sup>Institute of Agricultural Biology and Biotechnology, CNR, National Research Council, Via Moruzzi, 56124 Pisa, Italy

## Summary

Authors for correspondence:

Elena Loreti

Tel: +39 050 881902

Email: loreti@ibba.cnr.it

Pierdomenico Perata

Tel: +39 050 881901

Email: p.perata@santannapisa.it

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## Introduction

Plants, during their life, have to face several biotic and abiotic stresses that can compromise their growth. Although environmental conditions such as drought, flooding, salt and excessive heat represent abiotic constraints to plant growth and development, pathogens are biotic factors potentially influencing plant survival. Plant pathogens are divided into two main groups depending on their lifestyle. Biotrophs invade the host cells maintaining host viability and deriving nutrients from living cells, whereas necrotrophs derive their nutrients by killing the host plant. Among the latter group, *Botrytis cinerea* is one of the most invasive pathogens, causing grey mould disease on several crop plant species (Williamson *et al.*, 2007). Due to the huge damage that *B. cinerea* causes in terms of productivity, there is increasing interest in the mechanism(s) utilized by plants to counteract infection by this fungus. Plants rely on a sophisticated network of signal transduction pathways to respond to pathogen attacks, leading to metabolic and transcriptional reprogramming. In *Arabidopsis* the response against biotrophic pathogens predominantly is associated to salicylic acid (Wildermuth *et al.*, 2001), whereas the response to necrotrophic pathogens is typically mediated by jasmonate (JA) and ethylene (ET) signalling pathways that are transduced by APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors (Thaler *et al.*, 2004; Broekgaarden *et al.*, 2015; Huang *et al.*, 2015). One of the best

- Low oxygen availability often is associated with soil waterlogging or submergence, but may occur also as hypoxic niches in otherwise aerobic tissues. Experimental evidence assigns a role in *Botrytis cinerea* resistance to a group of oxygen-unstable Ethylene Response Factors (ERF-VII). Given that infection by *B. cinerea* often occurs in aerobic organs such as leaves, where ERF-VII stability should be compromised, we explored the possibility of local leaf hypoxia at the site of infection.
- We analyzed the expression of hypoxia-responsive genes in infected leaves. Confocal microscopy was utilized to verify the localization of the ERF-VII protein RAP2.12. Oxygen concentration was measured to evaluate the availability of oxygen (O<sub>2</sub>).
- We discovered that infection by *B. cinerea* induces increased respiration, leading to a drastic drop in the O<sub>2</sub> concentration in an otherwise fully aerobic leaf. The establishment of a local hypoxic area results in stabilization and nuclear relocalization of RAP2.12. The possible roles of defence elicitors, ABA and ethylene were evaluated.
- Local hypoxia at the site of *B. cinerea* infection allows the stabilization of ERF-VII proteins. Hypoxia at the site of pathogen infection generates a nearly O<sub>2</sub>-free environment that may affect the stability of other N-degron-regulated proteins as well as the metabolism of elicitors.

characterized ERF transcription factors involved in *B. cinerea* resistance is OCTADECANOID-RESPONSIVE ARABIDOPSIS 59 (ORA59). Plants overexpressing *ORA59* (35S:*ORA59*) showed enhanced resistance to *B. cinerea*, whereas *ORA59*-silenced plants were more strongly affected, indicating that this transcription factor is involved in the plant response to this pathogen (Pré *et al.*, 2008). Moreover, *ORA59* gene expression is induced synergistically by JA and ET confirming that ORA59 is an important player in the JA and ET signalling pathway (Pré *et al.*, 2008).

It was demonstrated recently that ORA59 interacts with RAP2.3 (Kim *et al.*, 2018), a transcription factor that, together with RAP2.12, RAP2.2, Hypoxia Responsive ERF (HRE)1 and 2, belongs to the subgroup VII of the ethylene transcription factors (ERF-VII). ERF-VII are master regulator genes for the tolerance to hypoxia through a complex mechanism of oxygen (O<sub>2</sub>)-dependent destabilization representing a sophisticated method for O<sub>2</sub> and nitric oxide (NO) sensing (Licausi *et al.*, 2011; Gibbs *et al.*, 2011, 2014). Under normoxia ERF-VIIs are degraded via the N-degron pathway (Varshavsky, 2011). The N-terminal methionine (Met) is cleaved leading to an exposed cysteine (Cys) which is oxidized by PLANT CYSTEINE OXIDASEs (PCOs; Weits *et al.*, 2014; White *et al.*, 2018) that convert Cys to Cys-sulfinic acid in a reaction requiring molecular O<sub>2</sub>. Arginylation catalyzed by ARGINYL tRNA TRANSFERASE (ATE) follows, inducing ubiquitination by the ligase PROTEOLYSIS 6 (PRT6),

and subsequent proteasomal degradation (26S proteasome). Under hypoxia, PCOs cannot oxidize the exposed Cys, and ERF-VII transcription factors are stabilized and drive transcriptional activation of the hypoxia-responsive genes (Bailey-Serres *et al.*, 2012; Gasch *et al.*, 2016; Gibbs *et al.*, 2011; Licausi *et al.*, 2011). All five ERF-VII transcription factors are involved in plant response to low O<sub>2</sub> conditions (Giuntoli & Perata, 2018). Intriguingly, it was demonstrated recently that RAP2.2 plays an important role against the infection to *B. cinerea*. Plants overexpressing *RAP2.2* (*35S:RAP2.2*) and a mutant line (*rap2.2-3*) showed higher and lower resistance, respectively, suggesting that RAP2.2 is important for Arabidopsis tolerance to *B. cinerea* (Zhao *et al.*, 2012).

This evidence highlighted the possibility that RAP2.2, RAP2.3 and possibly other ERF-VIIs, may represent key regulators both in the low-O<sub>2</sub> and plant pathogen responses. Remarkably, it was shown that the N-degron pathway positively regulates the biosynthesis of plant-defence metabolites such as glucosinolates, as well as the biosynthesis and response to JA in response to *Pseudomonas syringae* (de Marchi *et al.*, 2016). Additionally, the ERF-VII transcription factors enhance pathogen-induced stomatal closure (Vicente *et al.*, 2019). Vicente *et al.* (2019) results also indicated, differently from de Marchi *et al.*, (2016), that N-degron pathway mutants are more tolerant to *P. syringae* infection.

The involvement of the O<sub>2</sub>-destabilized ERF-VII proteins in the response of plants to biotic stresses raises the question about the mechanism by which they get stabilized during pathogen infection. Pathogens often attack fully aerobic tissues such as leaves and, additionally, they often induce the synthesis of NO that can further destabilize the ERF-VII proteins (Gibbs *et al.*, 2014). Given that these proteins are potentially highly unstable during pathogen infection, it is tempting to speculate that pathogen infection locally induces hypoxic conditions in an otherwise fully aerobic plant tissue such as leaves. Alternatively, a mechanism independent from O<sub>2</sub> might stabilize the ERF-VII proteins.

In this paper, we investigated the effects of *B. cinerea* on the hypoxic status of the infected leaves.

## Materials and Methods

### Plant material

Arabidopsis (*Arabidopsis thaliana*, Col-0 ecotype) was used. Other genotypes used were a line constitutively expressing a version of the RAP2.12 protein that also is stable in air (*35S:A-RAP2.12*; Licausi *et al.*, 2011); a line expressing  $\beta$ -glucuronidase (GUS) under the control of the *PLANT CYSTEINE OXIDASE* (*PCO1*) promoter (*pPCO1:GUS*; Weits *et al.*, 2014), and the quintuple *erfVII* mutant (ERF-VII, subgroup VII of ETHYLENE RESPONSE FACTOR) (Marín-de la Rosa *et al.*, 2014) which is a null mutant for *RAP2.2*, *RAP2.3*, *RAP2.12*, *Hypoxia Responsive ERF* (*HRE*) 1 and 2, and a knock-down for *RAP2.2* (Giuntoli *et al.*, 2017). The *phytoalexin deficient 3* (*pad3*) mutant was obtained from the Nottingham Arabidopsis Stock Center

(N3805). Plants were grown in pots for 3–4 wk at 23°C with a 12 h : 12 h, light : dark photoperiod at 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  intensity before being utilized for experiments.

### Plant inoculation with *B. cinerea*, *A. brassicicola* and *P. syringae*

The *Botrytis cinerea* strain (Ferrari *et al.*, 2003) was cultured on MEP plates (maltose extract 20 g l<sup>-1</sup>, peptone 10 g l<sup>-1</sup> and agar 15 g l<sup>-1</sup>) for 15–20 d at 23°C in the dark. Conidia were collected by washing the plates with potato dextrose broth (PDB, Formedium™) medium and the spore suspension was adjusted to the appropriate concentration ( $\sim 1 \times 10^6$ ). Inoculation with *B. cinerea* was conducted on 3–4-wk-old plants by placing 5  $\mu\text{l}$  droplets of a spore suspension ( $1 \times 10^6$  conidia ml<sup>-1</sup>) in 12 g l<sup>-1</sup> PDB on leaves (placing the drops on the sides of the rib). *Alternaria brassicicola* was cultured on potato dextrose agar (PDA, Formedium™) plates for 7–10 d at 23°C in the dark. Conidia were collected by washing the plates with potato dextrose broth (PDB) medium and the spore suspension was adjusted to the appropriate concentration ( $\sim 1 \times 10^6$ ). Inoculation with *B. cinerea* and *A. brassicicola* was conducted on 3–4-wk-old plants by placing 5  $\mu\text{l}$  droplets of a spore suspension ( $1 \times 10^6$  conidia ml<sup>-1</sup>) in 12 g l<sup>-1</sup> potato dextrose broth on leaves (placing the drops on the sides of the rib). *Pseudomonas syringae* (*Pst* DC3000) was cultured on nutrient agar (NAG) plates (Oxoid) for 2 d at 28°C in the dark. Plates were washed with 10 mM MgCl<sub>2</sub> containing Silwet L77 (0.05%). Plants were dipped in the *P. syringae*-containing solution with OD<sub>600</sub> of 0.2 (*c.* 10<sup>8</sup> colony forming units (CFU) ml<sup>-1</sup>). The inoculated plants were placed in plastic tanks with lids and the humidity was maintained by placing a steam source inside. Plants were incubated in a growth chamber (Percival Scientific, Perry, IA, USA) at 23°C with a 12 h : 12 h, light : dark photoperiod at 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  intensity for 24 h.

### Plant treatment with flagellin 22, OGs, 1-MCP and ABA

Plants were treated with flagellin 22 peptide (flg22; Genscript) at 1 or 10  $\mu\text{M}$ , with oligogalacturonides (OGs) (DP 10–15; Elicityl, Crolles, France) at 200  $\mu\text{g ml}^{-1}$ , and abscisic acid (ABA) at 0.1 or 1  $\mu\text{M}$  by placing two 5- $\mu\text{l}$  droplets on leaves which are incubated for 24 h, unless differently shown in figures. Plants were treated with 1-methylcyclopropene (1-MCP) to inhibit ethylene (ETH) signalling as follows. Plants were closed in a plastic box (4 l) in which 150 mg 1-MCP was placed in a beaker. Water (50 ml) was added rapidly and the box closed immediately. Plants were kept in the box for 1 h and subsequently utilized for the experiments.

### Total RNA extraction and qPCR

Total RNA was extracted as described previously (Perata *et al.*, 1997) with a minor modification (omission of aurintricarboxylic acid) to make the protocol compatible with the subsequent PCR procedures. Electrophoresis using a 1% agarose gel was performed for all RNA samples to check for RNA integrity, followed

by spectrophotometric quantification. cDNA was synthesized from 1 µg of total RNA using the Maxima Reverse Transcriptase kit (Life Technologies). Quantitative (q)PCR amplification was performed on 30 ng of cDNA with the ABI Prism 7300 sequence detection system (Applied Biosystems, Waltham, MA, USA), using the PowerUp SYBRGreen Master Mix (Applied Biosystems). *UBIQUITIN10* (*At4g053290*) was exploited as the house-keeping gene. Relative expression levels were calculated using GENORM (<https://genorm.cmgg.be/>). A full list of the primers used for qPCR is provided in Supporting Information Table S1.

### GUS staining

Histochemical GUS staining was carried out according to Jefferson *et al.* (1987). Briefly, plant material was fixed immediately after sampling in ice-cold 90% acetone for 1 h, rinsed several times in 100 mM phosphate buffer (pH 7.2), and then stained in a freshly prepared reaction solution (0.2% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide and 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide, sodium salt dissolved in DMSO) in 100 mM phosphate buffer, pH 7.2). Plants were stained overnight and chlorophyll was eliminated from green tissues by washing them with absolute ethanol.

### Laser scanning confocal microscopy and quantification of fluorescence intensity

RAP2.12-GFP fluorescence was imaged post-infection with *B. cinerea* using a Zeiss Airyscan 800 confocal microscope (GFP, green fluorescent protein). ZEN BLUE software (Carl Zeiss, Oberkochen, Germany) was used to quantify RAP2.12-GFP fluorescence intensity. Five individual biological replicates were used for each treatment. Each data point represents the average fluorescence intensity at the nuclei or plasma membrane of all analyzed cells in one biological replicate. Boxplot limits represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles of each dataset. The whiskers extend to the lowest and highest data point within the 1.5 × interquartile range of the 25<sup>th</sup> and 75<sup>th</sup> quartile. The central line represents the median.

### Ethylene measurement

Ethylene production was measured by enclosing a single whole plant, after having gently removed the substrate from the roots, in glass air-tight containers (30 ml) with holed plastic screw caps provided with rubber septa. Gas samples (2 ml) were taken from the headspace of the containers after 1 h incubation at room temperature. The ETH concentration in the sample was measured by a gas chromatograph (HP5890; Agilent Technologies Italia SpA, Cernusco sul Naviglio MI, Italy) using a flame ionization detector (FID), a stainless steel column (150 × 0.4 cm i.d. packed with HaySep<sup>®</sup>; Agilent Technologies, Santa Clara, CA, USA), column and detector temperatures of 70°C and 350°C, respectively, and nitrogen carrier gas at a flow rate of 30 ml min<sup>-1</sup>. Quantification of ETH was achieved by external standard techniques according to Mensuali Sodi *et al.* (1992) Data represent the mean of five biological replicates and ETH was expressed as pl g<sup>-1</sup> h<sup>-1</sup> FW.

### Oxygen measurement

Oxygen (O<sub>2</sub>) concentration was measured using a FireStingO2 high-precision, PC-controlled fibre-optic oxygen meter produced by Pyro Science. The needle-type oxygen probe used was OXR50. A MM33 micromanipulator (Unisense) was used to precisely insert the sensor into the leaf tissue. Air temperature was measured simultaneously using the TDIP15 temperature probe.

### Respiration measurement

The respiration rate was measured in excised leaves as described by Kerpen *et al.*, (2019). The fresh weight of the leaves was determined before the O<sub>2</sub> measurements. Oxygen consumption was measured with an integrated optical oxygen sensor in respiration vials (Pyroscience) filled with sterile distilled H<sub>2</sub>O and under continuous mixing using a magnetic stirrer. The O<sub>2</sub> consumption rate was calculated by dividing the decrease in O<sub>2</sub> concentration by the time and corrected for the weight of each sample.

### Statistics

Data were subjected to ANOVA and mean values were separated using Tukey's post-hoc test,  $P < 0.05$ . Values that significantly differ from each other are indicated by different letters in figures. Pairwise comparison were performed by Student's *t*-test.

## Results

### *Botrytis cinerea* infection generates local hypoxia in the leaf

Analysis of the response to *B. cinerea* in terms of induction of pathogen-responsive gene expression (*PDF1.2*) showed that infection by the fungus occurred under our experimental conditions (Fig. 1). To explore the possibility of hypoxia at the site of infection, we analyzed the mRNA level of several anaerobic genes in leaves that were infected with *B. cinerea* (Fig. 1). The induction was evident (> 3-fold induction) for most of the genes, particularly for *HRE2*, but it was limited for *ALCOHOL DEHYDROGENASE 1 (ADH1)* (Fig. 1). Dilution of RNA coming from uninfected areas with that from the infected area may influence the result, but dissection of leaves was proven to be difficult, owing to the fragility of the infected area. We thus decided to observe hypoxia-dependent gene induction using *pPCO1:GUS* plants, in which the promoter of *PCO1*, a gene which is strongly and specifically induced by hypoxia (Weits *et al.*, 2014), drives the expression of the GUS reporter gene. When infected with *B. cinerea*, leaves of *pPCO1:GUS* plants showed strong activation of the *PCO1* promoter as blue GUS staining corresponding to the sites where *B. cinerea* spores were applied (Fig. 2a). The GUS staining observed in control plants was due to the expression of hypoxia-responsive genes in the shoot apex and in young leaves (Giuntoli *et al.*, 2014).

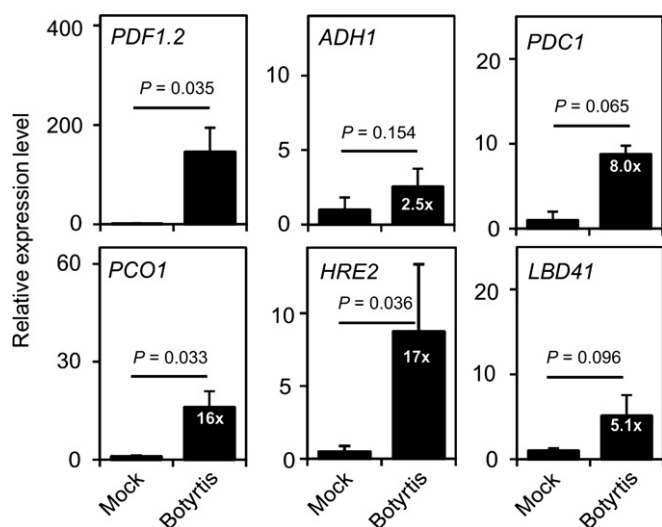
We directly measured the O<sub>2</sub> content at the sites of infection with an oxygen microsensor, finding that it drastically dropped

from a value of 18% in control leaves to < 2% in the infected area (Fig. 2b,c). The rate of respiration of infected leaves was more than three-fold higher, when compared to mock-treated leaves (Fig. 2d), suggesting that local hypoxia at the site of infection results from faster O<sub>2</sub> consumption. We verified if the hypoxia was reached faster in *pad3*, a mutant that is more susceptible to *B. cinerea*. The results showed that hypoxia was reached earlier in *pad3* compared to the wild-type, correlating well with the lesion size at two different times from infection in the two genotypes (Fig. 2e).

We explored the possibility that other pathogenic fungi or bacteria also could induce hypoxia during infection. As observed post-infection with *B. cinerea* (Fig. 2a), Arabidopsis leaves infected by *A. brassicicola*, a necrotrophic fungus, showed a very clear, localized induction of the *PCO1* promoter (Fig. S1a). Infection by a hemibiotrophic bacterium, *P. syringae*, did not, instead, induce hypoxia-related symptoms (Fig. S1b).

### Local hypoxia at the infection site results in stabilization of RAP2.12

Next we tested if hypoxia originating at the site of *B. cinerea* infection was sufficient for increased abundance and relocalization of RAP2.12 to the nuclei in an otherwise fully aerobic leaf. We used a *35S:RAP2.12-GFP* reporter line to visualize RAP2.12 in epidermal cells of leaves that were treated with broth-only (Mock) or infected with *B. cinerea*. Although mock-treated leaves predominantly showed membrane localization of RAP2.12-GFP, occasionally we also found GFP signal at the nuclei (Fig. 3a). However, *B. cinerea* inoculation led to a strong increase in RAP2.12-GFP fluorescence in the nuclei at the area of infection,



**Fig. 1** Infection with *Botrytis cinerea* results in induction of several anaerobic genes. *Botrytis cinerea* was inoculated as drops on leaves of 3–4-wk old Arabidopsis plants grown in soil (mock: culture broth). The entire rosette was harvested, extracted for RNA, and the resulting cDNA analyzed by quantitative PCR for the mRNA of the genes indicated in the figure. Data are mean  $\pm$  SD ( $n = 3$ ). The  $P$ -value resulting for pairwise comparison by Student's  $t$ -test is shown.

as well as in the area of the leaf that is close to the inoculation site (Fig. 3a,b). These results indicate successful RAP2.12 stabilization and an increase in overall RAP2.12 quantity, mostly in the nucleus-localized component, upon *B. cinerea*-induced hypoxia (Fig. 3a,b). We tested if ERF-VII proteins are important for contrasting infection by *B. cinerea*, as proposed by Zhao *et al.* (2012) by challenging the pentuple ERF-VII mutant (*erfvii*) and a line overexpressing a constitutively stable version of RAP2.12 (*35S: $\Delta$ -RAP2.12*) with *B. cinerea*. The results confirmed that ERF-VII proteins contribute to Arabidopsis tolerance to *B. cinerea*, as demonstrated by the larger lesion area observed in the *erfvii* mutant (Fig. S2a,b) and higher expression of the *B. cinerea* actin (*Bc-Actin*) gene in infected plants (Fig. S2c). Overexpression of a stable version of RAP2.12, however, did not enhance tolerance to the fungus (Fig. S2a,b).

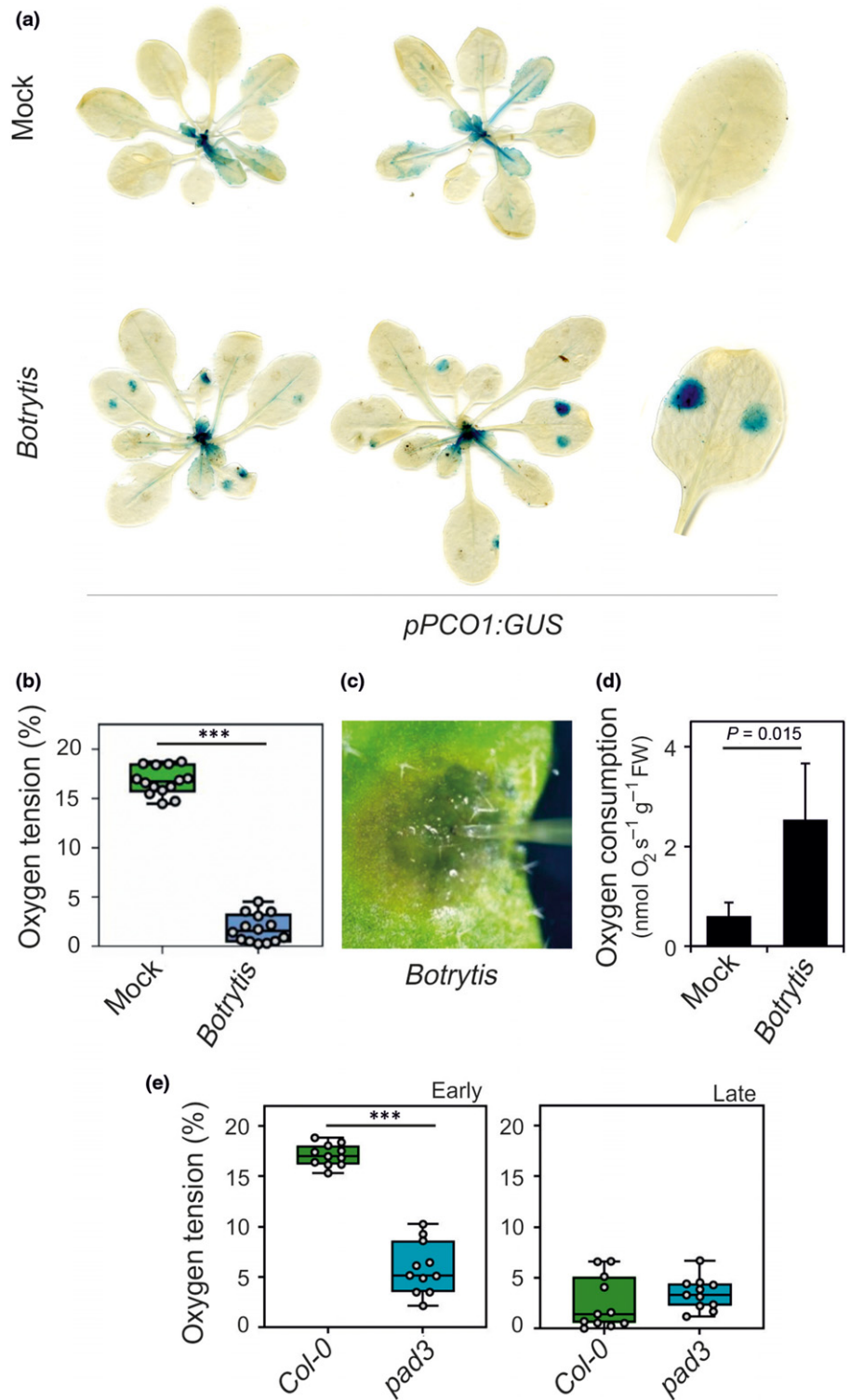
### Ethylene does not influence ERF-VII expression following *B. cinerea* infection

The *ERF-VII* genes are ETH-inducible (Hinz *et al.*, 2010) and ETH is produced during the infection by *B. cinerea* (Qadir *et al.*, 1997; Chague *et al.*, 2002; Zhu & Tian 2012). We confirmed ETH production 24 h post-infection with *B. cinerea* (Fig. 4a). Inhibition of ETH perception by 1-methylcyclopropene (1-MCP; see Fig. S3 for evidence of the efficacy of the inhibitor) reduced the expression of *PDF1.2* (Fig. 4b). However, 1-MCP did not affect the expression of *pPCO1:GUS* post-infection with *B. cinerea*, indicating that the ETH is dispensable for the activation of the anaerobic response (Fig. 4c). As we observed an overall increase of RAP2.12-GFP in the nuclei of *B. cinerea*-infected leaves (Fig. 3), we explored the possibility that infection with *B. cinerea* not only might lead to hypoxia-dependent stabilization of RAP proteins, but also to ETH-induced expression of these ERF-VII genes. However, we observed that most *ERF-VII* genes were not upregulated in infected leaves, with the notable exception of *HRE2* (Fig. 4d). The induction of *HRE2* was not influenced by 1-MCP, suggesting independence of this gene from ETH as observed previously (Hess *et al.*, 2011).

### Induction of anaerobic genes by *B. cinerea* is independent from wounding and ABA

We explored the possibility that infection by *B. cinerea* causes wounds in the leaf and that this could lead to induction of hypoxia-regulated genes. Wounding induces *ADH1* expression (Kato-Noguchi, 2001) and wounding triggers resistance to *B. cinerea* (Chassot *et al.*, 2008), making the link between wounding caused during *B. cinerea* infection and *ADH1* expression possible. We confirmed that wounding triggers expression of anaerobic genes, as indicated by GUS activity along mechanically-induced wounds in *pPCO1:GUS* plants (Fig. 5a). *HRE2* and *ADH1* expression was induced by wounding, whereas *PDF1.2* was not (Fig. 5b). The expression of an ABA-inducible gene, *RD29A* occurs as early as 1 h after wounding, suggesting that wounding induces the synthesis of ABA. Given that *ADH1* is induced by ABA (de Bruxelles *et al.*, 1996), it is likely that the expression of *ADH1* is the

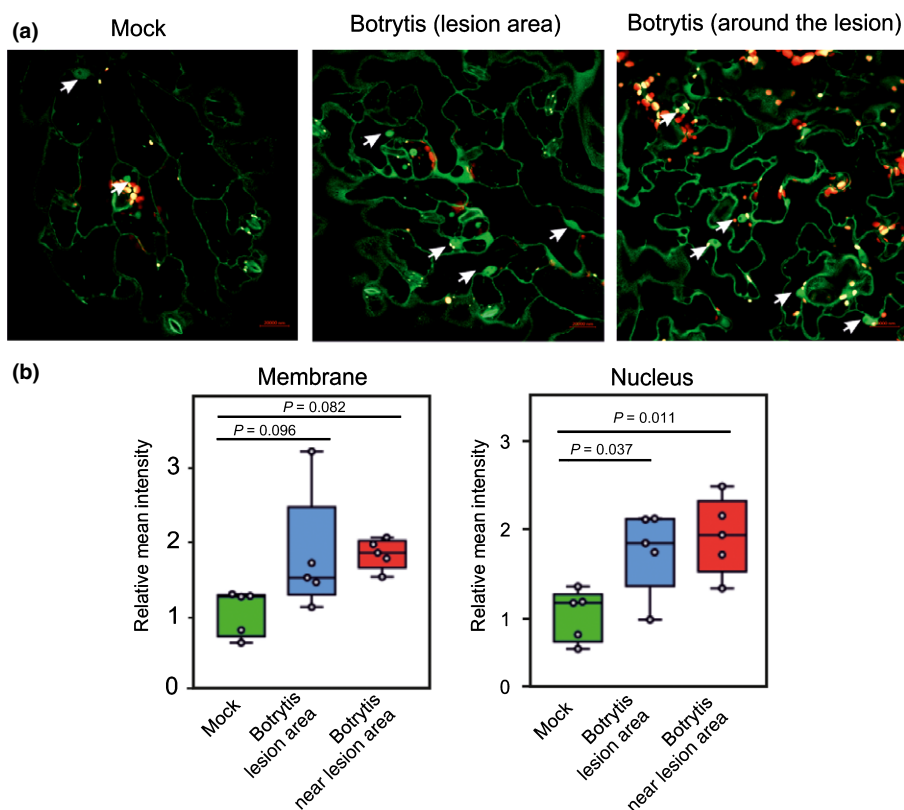
**Fig. 2** Infection by *Botrytis cinerea* results in localized hypoxia and expression of the transgenic line having the  $\beta$ -glucuronidase (GUS) gene under the control of the *PLANT CYSTEINE OXIDASE 1* (PCO1) promoter (pPCO1:GUS) reporter. *Botrytis cinerea* was inoculated as two drops on each leaves of Arabidopsis plants grown in soil (mock: culture broth). (a) GUS staining revealing the expression of the pPCO1:GUS reporter. (b) Oxygen tension measured using an oxygen microsensor probe (24 h post-infection). To assess the statistical significance of the observed datasets ( $n = 14$ ), one-way ANOVA was performed followed by Tukey's post-hoc test (\*\*\*,  $P < 0.001$ ). Lines in the boxes indicate the median, the bottom and top of each box denotes the first and third quartile, respectively, the dots represent the single data points and whiskers denote the min/max values. (c) Image showing the probe inserted in the centre of the infected area. (d) Oxygen consumption rate of infected leaves compared to mock-treated leaves (culture broth). Data are mean  $\pm$  SD ( $n = 5$ ). The  $P$ -value resulting for pairwise comparison by Student's  $t$ -test is shown. (e) Oxygen tension measured using an oxygen microsensor probe in wild-type plants and in *pad3* plants. Data were collected at an early and late stage of infection, visually evaluated from the lesion sizes and appearance. Lines in the boxes indicate the median, the bottom and top of each box denotes the first and third quartile, respectively, the dots represent the single data points and whiskers denote the min/max values. To assess the statistical significance of the observed datasets ( $n = 11$ ), one-way ANOVA was performed followed by Tukey's post-hoc test (\*\*\*,  $P < 0.001$ ).



consequence of wounding-induced ABA synthesis (Fig. 5b; Kato-Noguchi, 2001). However, although wounding induced an increased O<sub>2</sub> consumption in leaves, this was transient (Fig. 5c) and did not lead to hypoxia in the wounded leaf areas (Fig. 5d).

*Botrytis cinerea* is known to produce ABA (Marumo *et al.*, 1982; Izquierdo-Buono *et al.*, 2018). We checked if this

hormone contributes to the induction of anaerobic genes during *B. cinerea* infection. *pPCO1:GUS* leaves infected with *B. cinerea* showed GUS staining in the areas where the inoculum was placed (Fig. 6a). The effect of droplets of ABA applied to the leaves on the expression of GUS in *pPCO1:GUS* leaves was limited, but present (Fig. 6a). Furthermore, *ADH1* expression was clearly



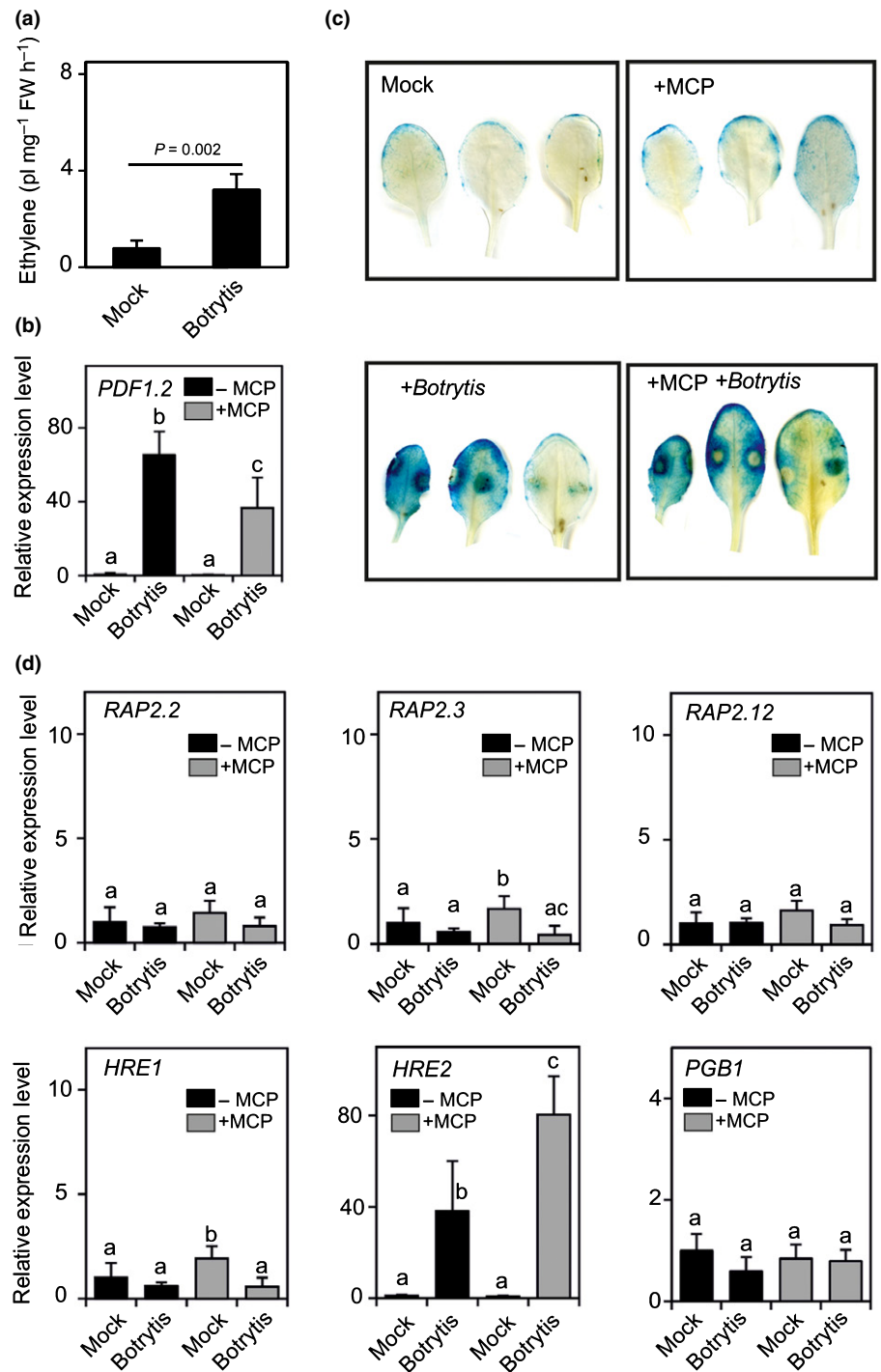
**Fig. 3** Infection by *Botrytis cinerea* results in increased signal and nuclear localization of RAP2.12:GFP (GFP, green fluorescent protein). (a) Representative confocal microscopy images of RAP2.12-GFP (green) and chlorophyll fluorescence (red) in *Arabidopsis* epidermal leaf cells in the *B. cinerea* lesion area or in proximity of the infected region. The leaf area directly under a drop of culture broth (mock) was used as control. (b) Relative mean intensity ( $n = 5$ ) of RAP2.12-GFP fluorescence as shown in (a) in the membrane or nucleus following mock or *B. cinerea* treatments. Lines in the boxes indicate the median, the bottom and top of each box denotes the first and third quartile, respectively, the dots represent the single data points and whiskers denote the min/max values. To assess the statistical significance of the observed datasets, one-way ANOVA was performed followed by Tukey post-hoc test.

induced by ABA, whereas it had no effect of *HRE2* expression (Fig. 6b). The expression of *RD29A*, a gene responding strongly to ABA, showed very high levels in the ABA-treated leaves but its expression was negligible in leaves infected with *B. cinerea* (Fig. 6b), indicating that *B. cinerea* infection does not lead to markedly increased ABA levels in the infected tissue. The  $O_2$  concentration at the sites where ABA was applied on the leaves was unaffected, as evident from the oxygen microsensor measurements (Fig. 6c). Overall, these results showed that, although ABA can induce *ADH1*, the contribution of ABA produced by the *B. cinerea* pathogen to the induction of anaerobic genes is negligible.

#### Elicitors of plant defence moderately decrease $O_2$ availability but cannot induce anaerobic genes

The induction of hypoxia at the site of *B. cinerea* infection raises the question about the trigger that leads to a marked induction of respiration and a concomitant reduction in oxygen availability. We verified if elicitation of plant defence is enough to induce the low  $O_2$  condition associated with *B. cinerea* infection. The bacterial flagellin peptide elicitor flg22 enhances resistance to *B. cinerea* (Ferrari *et al.*, 2007) and induces several pathogenesis-related genes (Denoux *et al.*, 2008). We treated *Arabidopsis* plants with flg22 and observed induction of *PDF1.2* but not of *ADH1* and *HRE2* (Fig. 7a). GUS staining was absent in *pPCO1:GUS* leaves treated with flg22, whereas *B. cinerea* was able to induce GUS expression as also shown Fig. 2a, but also Fig. 4c and 6a (Fig. 7b). Only when used at

10  $\mu$ M, flg22 was able to trigger a moderate decrease in  $O_2$  availability, from *c.* 15% in control leaves to <10% in flg22-treated leaves, an  $O_2$  concentration still too high to induce the expression of anaerobic genes (Fig. 7c). A time-course with 10  $\mu$ M flg22 confirmed that this elicitor is unable to trigger hypoxia even with shorter or longer treatment times (Fig. S4). We next explored the possibility that signalling originating from fragments of pectin (OGs) produced by the degradation of the host cell wall may induce hypoxia in the leaves of *B. cinerea*-infected plants. OGs activate plant innate immunity by functioning as damage-associated molecular patterns and induce several pathogenesis genes, such as *PAD3* (Ferrari *et al.*, 2007). We failed to observe induction of *PAD3* when the length of the treatment was >24 h (not shown) and decided to check the plant's response to OGs in a time-course experiment. The results showed that *PAD3* is induced after 10 h from the start of OG treatment (Fig. 7d). However, *ADH1* and *HRE2* were not induced at any of the analyzed time points (Fig. 7d). Analysis of the  $O_2$  content in the leaves indicated that leaves responding to OGs are fully aerobic (Fig. 7e) and the *pPCO1:GUS* leaves did not show any sign of GUS activity (Fig. 7f). Finally, we used heat-killed mycelium as an elicitor, confirming also in this case the lack of hypoxia following the treatment, which was able to induce expression of defence-related genes but not the hypoxia-related ones (Fig. S5). Overall, these results indicated that elicitation of the defence pathway in the plant cells is not sufficient for induction of local hypoxia at the site of infection, suggesting that the metabolic/eliciting activity of the living fungus is responsible for the increase in  $O_2$  consumption.

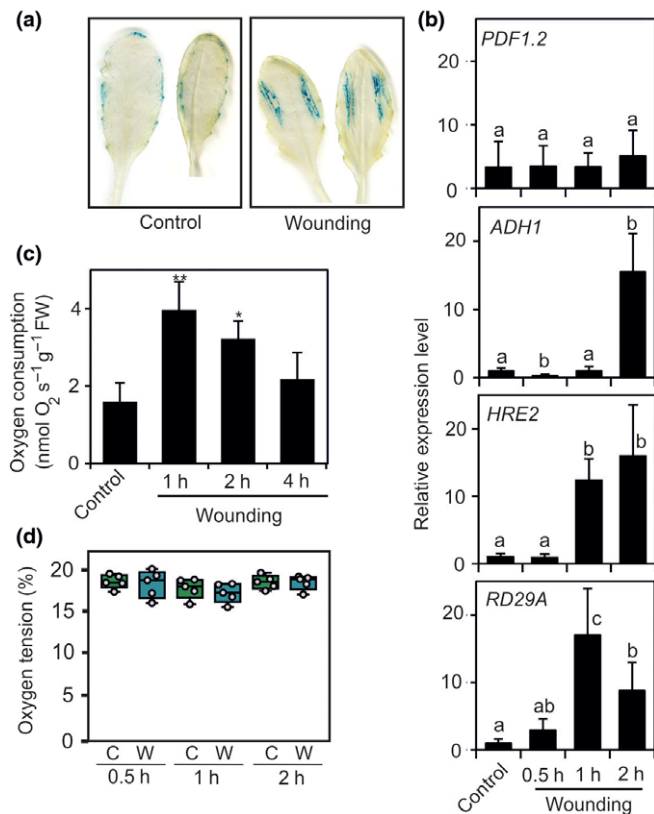


**Fig. 4** Ethylene production during *Botrytis cinerea* infection does not influence subgroup VII of ETHYLENE RESPONSE FACTOR (*ERF-VII*) expression. (a) Ethylene (ETH) production by plants infected with *B. cinerea* compared to mock-treated plants (culture broth). Data are mean  $\pm$  SD ( $n = 5$ ). The  $P$ -value resulting for pairwise comparison by Student's  $t$ -test is shown. (b) *PDF1.2* expression in leaves infected with *B. cinerea* compared to mock-treated leaves (culture broth). A set of plants was treated with 1-methylcyclopropene (1-MCP) to inhibit ETH signalling. Data are mean  $\pm$  SD ( $n = 5$ ). Differences ( $P < 0.05$ ; two-way ANOVA) are indicated by different letters. (c) GUS staining revealing the expression of the transgenic line having the  $\beta$ -glucuronidase (GUS) gene under the control of the *PLANT CYSTEINE OXIDASE 1* (*PCO1*) promoter (p*PCO1*:GUS) reporter. A set of plants was treated with 1-MCP to inhibit ETH signalling. (d) Expression of *ERF-VII* genes in leaves infected with *B. cinerea* compared to mock-treated leaves. A set of plants was treated with 1-MCP to inhibit ETH signalling. Data are mean  $\pm$  SD ( $n = 5$ ). Differences ( $P < 0.05$ ; two-way ANOVA) are indicated by different letters.

## Discussion

Subgroup VII of the ethylene transcription factors (*ERF-VII*) proteins are key components of the oxygen ( $O_2$ ) sensing mechanism in plants (Bailey-Serres *et al.*, 2012). Under hypoxia the low concentration of  $O_2$  permits the stabilization of these proteins, which accumulate in the nucleus to activate the hypoxia-responsive gene network. Besides their well-established role in hypoxia signalling (Loreti *et al.*, 2016), *ERF-VII* proteins also contribute to pathogen resistance (Zhao *et al.*,

2012; Gravot *et al.*, 2016; Kim *et al.*, 2018; Vicente *et al.*, 2019). Given the instability of *ERF-VII* proteins under aerobic conditions, their involvement in pathogen resistance implies that they can get stabilized in the plant's infected tissues. This may occur if hypoxia is established during pathogen infection or by modulation of nitric oxide (NO), the other important determinant for *ERF-VII* stability (Gibbs *et al.*, 2014; Gravot *et al.*, 2016), possibly by the NO-scavenging activity of the ethylene (ETH)-induced *PHYTOGLOBIN1* (*PGB1*; Hartman *et al.*, 2019).



**Fig. 5** Wounding-dependent induction of anaerobic genes is hypoxia-independent. (a)  $\beta$ -glucuronidase (GUS) staining revealing the expression of the transgenic line having the GUS gene under the control of the *PLANT CYSTEINE OXIDASE 1* (PCO1) promoter (*pPCO1:GUS*) reporter in wounded leaves. (b) Expression of *PDF1.2*, *ADH1*, *RD29A* in Arabidopsis leaves that were wounded. Sampling was as shown in the figure. Data are mean  $\pm$  SD ( $n = 5$ ). Differences ( $P < 0.05$ ; one-way ANOVA) are indicated by different letters. (c) Oxygen consumption of infected leaves compared to mock-treated leaves (culture broth). Data are mean  $\pm$  SD ( $n = 5$ ). Pairwise comparison by Student's *t*-test: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (d) Oxygen tension measured using an oxygen microsensor probe. Lines in the boxes indicate the median, the bottom and top of each box denotes the first and third quartile, respectively, the dots represent the single data points and whiskers denote the min/max values. Differences were not significant for  $P < 0.05$ .

Induction of hypoxia-responsive genes was indeed reported in roots infected by *Plasmiodiophora brassicae* (Gravot *et al.*, 2016) or upon *Agrobacterium tumefaciens* infection in stems (Kerpen *et al.*, 2019). Hypoxia in clubroot and in crown galls may result from slower O<sub>2</sub> diffusion rate in tumorigenic tissues (Jubault *et al.*, 2013) or from an intracellular competition for O<sub>2</sub> between the pathogen and the host (Gravot *et al.*, 2016). It also has been postulated that increased respiration rates to fuel rapid cell proliferation rates may cause hypoxia in the case of crown galls (Kerpen *et al.*, 2019), although O<sub>2</sub> consumption rate did not increase in clubroot tissue.

To date, there is no evidence of hypoxic condition in leaves infected by nontumorigenic pathogens like *Botrytis cinerea* apart from a transient induction of *ALCOHOL DEHYDROGENASE 1* (*ADH1*) in leaves infected by the hemibiotroph pathogen *Pseudomonas syringae* (Vicente *et al.*, 2019). However, this could

have resulted from synthesis of abscisic acid (ABA) occurring in *Pseudomonas*-infected leaves (de Torres-Zabala *et al.*, 2007), which induces *ADH1* expression (de Bruxelles *et al.*, 1996).

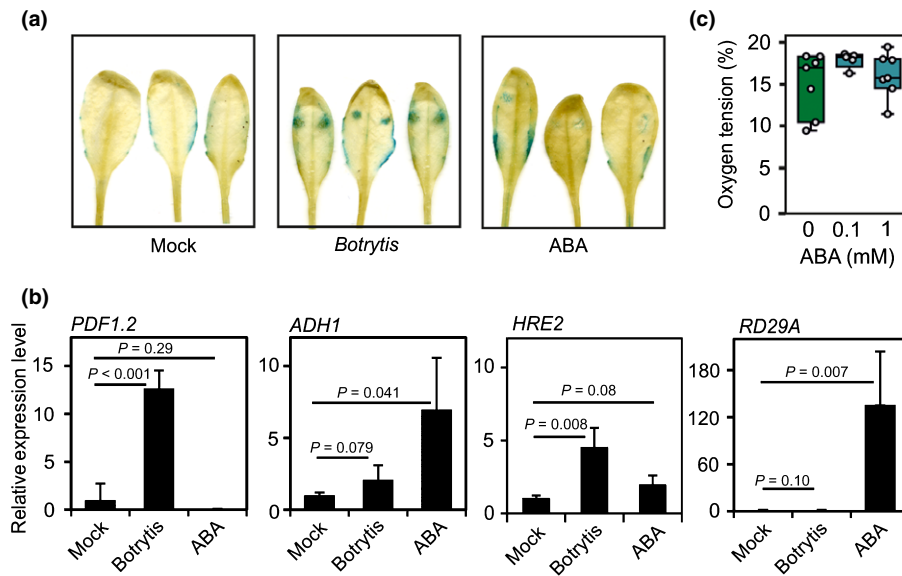
Although there is evidence linking ERF-VII proteins to *B. cinerea* resistance (Zhao *et al.*, 2012), the potential role of ERF-VIIs in aerobic tissues such as leaves is thus actually challenged by their instability if O<sub>2</sub> and/or NO are present. An environment characterized by high O<sub>2</sub> and NO concentrations is not compatible with RAP2.3 stability, making its interaction with OCTADECANOIC-RESPONSIVE ARABIDOPSIS 59 (ORA59) impossible (Kim *et al.*, 2018). Likewise, it was unclear how an unstable RAP2.2 can contribute to resistance to *B. cinerea* (Zhao *et al.*, 2012). Vicente *et al.* (2019) demonstrated a role for ERF-VII in stomatal defence against *Pseudomonas* – therefore in an anaerobic tissue. Also in this case the instability of ERF-VII would compromise their action.

Here, we demonstrated that infection by *B. cinerea* and *Alternaria brassicicola*, but not *P. syringae*, leads to the rapid establishment of localized hypoxia in an otherwise fully aerobic leaf. In the case of *B. cinerea*, this occurs though enhanced respiration at the site of infection (Fig. 2d), which locally depletes O<sub>2</sub> availability (Fig. 2b) at a level compatible with ERF-VII (RAP2.12) stabilization and accumulation in the plant's nuclei (Fig. 3a,b). It remains to be elucidated if the absence of hypoxia at the site of *P. syringae* infection is related to the plant's response to the pathogen in terms of respiratory metabolism. Hypoxia following infection by necrotrophic fungi also may arise from the tissue waterlogging in the necrotic area, although the increased respiration observed in *Botrytis*-infected leaves suggests that hypoxia is generated by a sequence of intense respiratory mechanisms at the site of infection. The establishment of hypoxia correlates with the plant's susceptibility to *B. cinerea* infection, as demonstrated by the faster hypoxia establishment in the *pad3* mutant (Fig. 2e). Although flagellin peptide (flg)22 and oligogalacturonides (OGs) do not induce hypoxia (Fig. 7b,e), it is very likely that other signalling molecules released by *B. cinerea* enhance the plant's respiration (Fig. 2d). This is supported by the evidence of enhanced respiration in Arabidopsis cells that were cultured in a millicell culture insert that enables molecular communication between the fungus and the plant cells without physical contact (Veillet *et al.*, 2017). Water-soaking of the leaf tissues infected by *B. cinerea* also may contribute to generating hypoxia.

Given the drastic decline in O<sub>2</sub> concentration at the sites where the infection occurs we discounted the possibility of an indirect effect of *B. cinerea* triggering a low-pH environment on components of the O<sub>2</sub> sensing machinery such as the PCO enzymes, which display reduced activity at pH < 7 (White *et al.*, 2018).

Wounding induced by penetration of *B. cinerea* in the plant's cells could potentially contribute to establishing local hypoxia in the leaves, but the strong induction of the GUS gene under the control of the *PLANT CYSTEINE OXIDASE 1* (PCO1) promoter (*pPCO1:GUS*) in mechanically wounded leaves (Fig. 5a) is likely ABA-dependent and uncoupled from the *B. cinerea*-dependent effect (Fig. 5b). ABA can indeed induce *ADH1* (Fig. 6b; de Bruxelles *et al.*, 1996) and wounding causes an ABA-dependent response that we observed in terms of *RD29A* expression





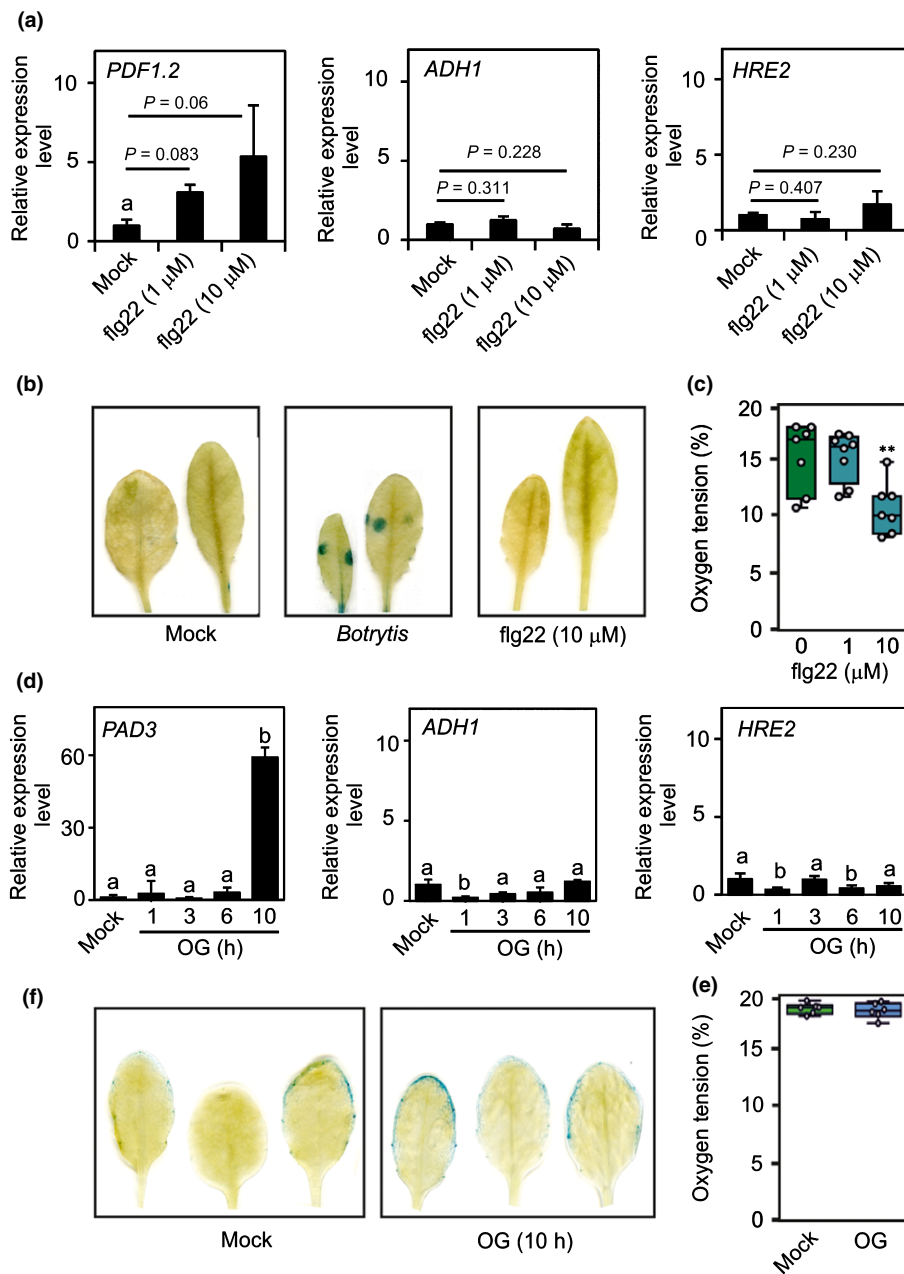
**Fig. 6** Abscisic acid (ABA) does not induce hypoxia. (a)  $\beta$ -glucuronidase (GUS) staining revealing the expression of the transgenic line having the GUS gene under the control of the *PLANT CYSTEINE OXIDASE 1* (PCO1) promoter (pPCO1:GUS) reporter in *Botrytis cinerea*-infected leaves and, to a minor extent, in ABA-treated leaves. (b) Expression of *PDF1.2*, *ADH1*, *RD29A* in Arabidopsis leaves that were infected with *B. cinerea* or treated with ABA. Data are mean  $\pm$  SD ( $n = 5$ ). Differences ( $P < 0.05$ ; one-way ANOVA) are indicated by different letters. (c) Oxygen tension measured using an oxygen microsensor probe. Lines in the boxes indicate the median, the bottom and top of each box denotes the first and third quartile, respectively, the dots represent the single data points and whiskers denote the min/max values. Differences were not significant for  $P < 0.05$ .

(Fig. 5b). But these symptoms of ABA signalling were absent in leaves infected with *B. cinerea* (Fig. 6b) and ABA did not induce *Hypoxia Responsive ERF 2* (*HRE2*) (Fig. 6b), indicating that, although *B. cinerea* may cause an increase in the leaf ABA content (Marumo *et al.*, 1982), this is insufficient to trigger *RD29A* expression and an ABA-dependent response on hypoxic genes such as *ADH1* (Fig. 6b).

Ethylene production during *B. cinerea* infection (Chaqué *et al.*, 2002) could have two consequences. On the one hand, it may contribute to induce the expression of *ERF-VII* at the transcriptional level (Hinz *et al.*, 2010; Hartman *et al.*, 2019), and on the other, it could enhance *ERF-VII* stability before hypoxia by increasing expression of the NO-scavenger *PGB1* (Hartman *et al.*, 2019). We observed production of ETH in the plants infected with *B. cinerea* (Fig. 4a), but we could not observe induction of *ERF-VII* genes, with the exception of a strong induction of *HRE2* (Fig. 4d). However, although *HRE1* is induced by ETH, *HRE2* is not (Hess *et al.*, 2011). This suggests that upregulation of *HRE2* was triggered upon *B. cinerea*-induced hypoxia and was independent of ETH. This is supported by the fact that the ETH signalling inhibitor 1-MPC (Mitochondrial Pyruvate Carrier) did not affect the induction of *HRE2* (Fig. 4d). Overall, given that the ETH-responsive *ERF-VII*s (Hinz *et al.*, 2010; Hess *et al.*, 2011) are not induced by *B. cinerea* infection and that *HRE2* induction is ETH-independent, we conclude that the amount of ETH produced during *B. cinerea* infection is not sufficient to affect the induction or stabilization of *ERF-VII*. In line with the absence of a marked ETH signalling pathway connecting with hypoxia sensing, *PGB1* was not induced by *B. cinerea* infection (Fig. 4d).

Oligogalacturonides are released during interaction with *B. cinerea*, which secretes polygalacturonase to degrade pectin. Those OGs with a degree of polymerization between 10 and 25 act as elicitors that contribute to trigger immunity against the fungal pathogen. Interestingly, OGs were found to induce NO production (Rasul *et al.*, 2012), which is known to negatively affect *ERF-VII* stability (Gibbs *et al.*, 2014). In several cases, we measured  $O_2$  concentrations close to anoxia at the site of *B. cinerea* infection (Fig. 2b), indicating that the amount of  $O_2$  available for PCOs activity is very low. Under these conditions the contribution of NO to *ERF-VII* stability is presumably marginal; however, we did observe a decrease in the aerobic expression of *ADH1* and *HRE2* after the addition of OGs (Fig. 7d), suggesting that the release of OGs during *B. cinerea* infection may indeed negatively influence *ERF-VII* stability.

The advantages of establishing hypoxia at the site of *B. cinerea* infection can be multiple. The fate of OGs as elicitors might depend on the establishment of local hypoxia at the site of *B. cinerea* infection. It was indeed recently discovered that OGs can get oxidized by a group of four Arabidopsis berberine bridge enzyme-like proteins, named OGOX1–4 (Benedetti *et al.*, 2018). Oxidized OGs display a reduced capability of activating the immune responses and are less hydrolysable by fungal polygalacturonases (Benedetti *et al.*, 2018). In the context of the dialog between the pathogen and the host, the  $O_2$ -dependent activity of OGOXs is potentially unfavourable for the plant because of the consequent reduced activity of OGs as elicitors. From another perspective, the fungus might induce hypoxia to maintain the OGs in a nonoxidized status and therefore metabolizable by the fungus itself. This latter possibility is more likely, given that overexpression of OXOGs results in enhanced resistance to *B. cinerea* (Benedetti *et al.*, 2018). Therefore, the *B. cinerea*-induced



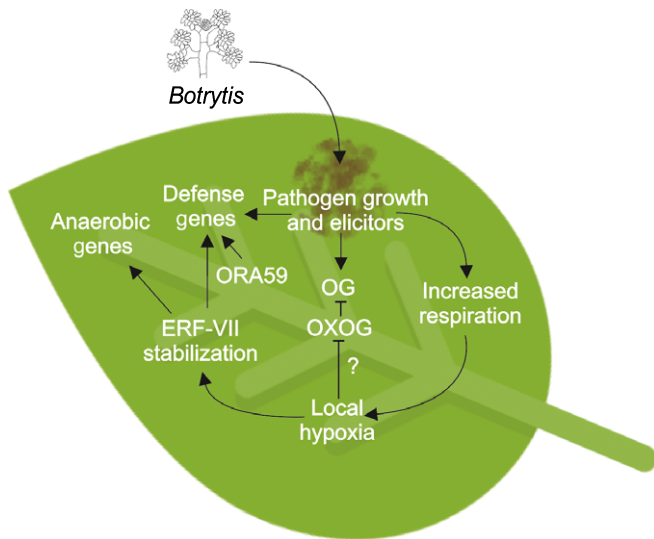
**Fig. 7** Elicitors of plant defence responses do not induce hypoxia. (a) Expression of *PDF1.2* and *ADH1* in Arabidopsis leaves that were infected with *Botrytis cinerea* or treated with flagellin peptide elicitor flg22. Data are mean  $\pm$  SD ( $n = 5$ ). The elicitor was applied as two droplets/leaf. The  $P$ -value resulting for pairwise comparison by Student's  $t$ -test is shown. (b)  $\beta$ -glucuronidase (GUS) staining revealing the expression of the transgenic line having the GUS gene under the control of the *PLANT CYSTEINE OXIDASE 1* (PCO1) promoter (pPCO1:GUS) reporter in *B. cinerea*-infected leaves but not in flg22-treated leaves. The elicitor was applied as two droplets/leaf. (c) Oxygen tension measured using an oxygen microsensor probe. Lines in the boxes indicate the median, the bottom and top of each box denotes the first and third quartile, respectively, the dots represent the single data points and whiskers denote the min/max values. Significant differences: \*\*,  $P < 0.01$ . (d) Expression of *PAD3* and *ADH1* in Arabidopsis leaves that were infected with *B. cinerea* or treated with oligogalacturonides (OGs) for the time indicated in figure. The elicitor was applied as two droplets per leaf. Data are mean  $\pm$  SD ( $n = 5$ ). Differences ( $P < 0.05$ ; one-way ANOVA) are indicated by different letters. (e) Oxygen tension measured using an oxygen microsensor probe. Lines in the boxes indicate the median, the bottom and top of each box denotes the first and third quartile, respectively, the dots represent the single data points and whiskers denote the min/max values. Differences were not significant for  $P < 0.05$ . (f) GUS staining revealing the absence of expression of the pPCO1:GUS reporter in OG-treated leaves. The elicitor was applied as two droplets per leaf.

localized hypoxia at the site of infection may provide a means to avoid oxidation of OGs and thus allow their metabolism by the fungus (Fig. 8).

The establishment of hypoxia at the site of *B. cinerea* infection also may promote pathogen resistance because it leads to the stabilization of ERF-VII proteins (Fig. 3). This will allow RAP2.3 to form a nucleus-localized complex with ORA59 to regulate plant defence genes (Kim *et al.*, 2018). RAP2.2 would be stabilized as well, contributing to the plant's resistance to *B. cinerea* (Zhao *et al.*, 2012). We indeed observed increased susceptibility to *B. cinerea* infection in a pentuple mutant (*erfvi*) defective in all five ERF-VII proteins (Fig. S2). This suggests that the establishment of hypoxia is part of plant defence response rather than a mere consequence of pathogen infection. Furthermore, all three RAP-type ERF-VIIs

(RAP2.2, RAP2.3, RAP2.12) will activate the hypoxia-responsive genes to protect the plant tissue from the negative consequences of hypoxia. Induction of *HRE2* by *B. cinerea* infection, and an *ERF-VII* gene which is strongly and stably induced under low- $\text{O}_2$  conditions, ensures that the transcription of hypoxic genes is steadily maintained (Licausi *et al.*, 2010). Enhanced stabilization of ERF-VII, as in *35S:A-RAP2.12* plants, however, does not enhance tolerance to *B. cinerea* (Fig. S2), suggesting also that a hypoxic pre-treatment would be ineffective for enhancing *B. cinerea* tolerance. A model explaining the mechanism of hypoxia induction and its relations with the plant's response to *B. cinerea* is reported in Fig. 8.

Hypoxia occurs in plants as a consequence of abiotic constraints such as soil waterlogging or flooding, but also in plant tissues and organs whose compactness limits  $\text{O}_2$  diffusion (van



**Fig. 8** Model explaining the establishment of local hypoxia at the sites of *Botrytis cinerea* infection. *Botrytis cinerea* infection results in increased respiration, inducing hypoxic conditions that lead to subgroup VII of ETHYLENE RESPONSE FACTOR (ERF-VII) stabilization and translocation to the nucleus. Here, these transcription factors induce the expression of genes required for hypoxia adaptation as well as, together with OCTADECANOID-RESPONSIVE ARABIDOPSIS 59 (ORA59) (Kim *et al.*, 2018) and other still nonidentified partners, genes involved in plant defence. Hypoxia may also influence the fate of oligogalacturonides (OGs), that are oxidized by OXOG in a reaction requiring molecular oxygen (Benedetti *et al.*, 2018).

Dongen & Licausi, 2015). Local hypoxic niches influence shoot apical meristem development (Weits *et al.*, 2019) as well as lateral root growth (Eysholdt-Derzso & Sauter, 2017; Eysholdt-Derzso & Sauter, 2019; Shukla *et al.*, 2019). We demonstrated here that not only tumorigenic root tissues (Jubault *et al.*, 2013) but also leaves develop hypoxia as a consequence of pathogen infection. This indicates that pathogen infection by itself can be sufficient to induce hypoxia and may be a common feature of biotic stresses. These sources of evidence expand the influence of hypoxia in plant growth and development well beyond plant's tolerance to flooding (Bailey-Serres & Voesenek, 2008). Besides ERF-VII proteins, the hypoxia-dependent stabilization of other proteins that are target of the Cys-branch of the N-degron pathway (Millar *et al.*, 2019) may influence pathogen resistance pathways, an area of study certainly deserving further work.

## Acknowledgements







The *B. cinerea* strain was kindly provided by Simone Ferrari (La Sapienza, University of Rome). We thank Benedetta Mattei (L'Aquila University) for useful discussion and Sabrina Sarrocco (University of Pisa) for advice on *B. cinerea* cultures management and for providing us with the *Alternaria brassicicola* strain.

## Author contributions

PP and EL conceived the work and devised the experimental design; MCV, EL and GN carried out the experimental work

and data analysis; DAW performed the confocal microscopy and oxygen measurement experiments; AM performed the ETH assays; PP and EL wrote the manuscript and all authors reviewed and commented on the manuscript.

## ORCID

Elena Loreti  <https://orcid.org/0000-0002-5255-4983>  
 Anna Mensuali  <https://orcid.org/0000-0002-3983-9124>  
 Giacomo Novi  <https://orcid.org/0000-0003-4113-6345>  
 Pierdomenico Perata  <https://orcid.org/0000-0001-9444-0610>  
 Maria Cristina Valeri  <https://orcid.org/0000-0002-0669-4053>  
 Daan A. Weits  <https://orcid.org/0000-0003-4423-5568>

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Infection by *Alternaria brassiciola*, but not *Pseudomonas syringae* (Pst DC3000), results in localized hypoxia and expression of the pPCO1:GUS reporter.

**Fig. S2** Effects of *B. cinerea* infection on wild-type plants (*Col-0*), the pentuple *ERFVII* mutant (*erfvii*) and a line overexpressing a constitutively stable version of RAP2.12 (*35S:A-RAP2.12*).

**Fig. S3** Effects of *MCP* on Arabidopsis plants.

**Fig. S4** Effects of flg22 in a time-course experiment.

**Fig. S5** Effects of heat-killed mycelium on Arabidopsis plants.

**Table S1** Primers used for gene expression analysis using real-time quantitative RT-PCR.

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