Plant Physiology Preview. Published on October 4, 2012, as DOI:10.1104/pp.112.200154

RUNNING TITLE: ABA-mediated regulation of Arabidopsis basal resistance

Corresponding Author: Antonio Molina

Address: Centro de Biotecnología y Genómica de Plantas (UPM-INIA),

Universidad Politécnica de Madrid, Campus de Montegancedo,

E-28223-Pozuelo de Alarcón (Madrid), Spain.

Phone: (34) 913364552

Fax. (34) 917157721

e-mail: antonio.molina@upm.es

Journal Research Area: Plant Interacting with Other Organisms

Disruption of abscisic acid signaling constitutively activates Arabidopsis resistance to the necrotrophic fungus *Plectosphaerella cucumerina*

Andrea Sánchez-Vallet[1,2*], Gemma López[1,2], Brisa Ramos[1,2], Magdalena Delgado-Cerezo[1,2], Marie-Pierre Riviere[1,2], Francisco Llorente[1,2], Paula Virginia Fernández[3], Eva Miedes[1,2], José Manuel Estevez[3], Murray Grant[4] and Antonio Molina[1,2¶].

[1]Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Universidad Politécnica de Madrid (UPM), Campus de Montegancedo, E-28223-Pozuelo de Alarcón (Madrid), Spain.

[2]Departamento de Biotecnología, Escuela Técnica Superior Ingenieros Agrónomos, UPM, E-28040-Madrid, Spain.

[3]Catedra de Quımica de Biomoleculas, Departamento de Biología Aplicada y Alimentos (CIHIDECAR-CONICET), Facultad de Agronomıa, Universidad de Buenos Aires, Av. San Martın 4453, C1417DSE Buenos Aires, Argentina

[4]College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, United Kingdom.

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[¶]Corresponding Author: A. Molina (antonio.molina@upm.es)

Financial source: This work was supported by Spanish MICINN (Grants BIO2009-07161 and EUI2008-03728/BALANCE to A. Molina).

[*]**Current address A. S. -V.:** Laboratory of Phytopathology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

Key Words: abscisic acid, cell wall, hormone balance, necrotropic fungi, plant immunity.

Total word count (9654): Abstract (246), Introduction (1089), Results (3497), Discussion (1715), Methods (1536), Acknowledgment (53), Table (290) and Legend to Figures (1228).
Total characters (with spaces) including Literature Cited: 85,125
Total Figure number: 7, 5 in colour
Total Tables: 1
Supplementary Material: 10, 6 Figures and 7 Tables

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiology.org) is: Dr. Antonio Molina (antonio.molina@upm.es).

Contributions of Authors:
Andrea Sánchez-Vallet: wrote the manuscript, collected the data included in Figures 1-5 and 7, in Supplemental Figures 1, 3-5, and in the Tables.
Gemma López: selected the double mutants, performed qRT-PCR included in Figures 1 and 4.
Brisa Ramos: collected the Supplemental Figure 1 data
Magdalena Delgado-Cerezo: collected the Supplemental Figure 2 data.
Marie-Pierre Riviere: collected the Supplemental Figure 3 data
Francisco Llorente: obtained the first data on ABA-deficient mutants resistance to necrotrophic fungi (Figure 1), and on the effect of ABA treatment in the resistance of wild-type and ABA-deficient mutants.
Paula Virginia Fernández: collected the data on cell wall determinations (Figure 6)
Eva Miedes: collected the data on cell wall determinations (Supplemental Figure 6)
José Manuel Estevez: wrote the manuscript, managed cell wall determinations, PI
Murray Grant: wrote the manuscript, managed hormone determinations, PI
Antonio Molina: wrote the manuscript, managed all aspect of the project, PI

Abstract

Plant resistance to necrotrophic fungi is regulated by a complex set of signaling pathways that includes those mediated by the hormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA). The role of ABA in plant resistance remains controversial as positive and negative regulatory functions have been described depending on the plant-pathogen interaction analysed. Here, we show that ABA signaling negatively regulates *Arabidopsis thaliana* resistance to the necrotrophic fungus *Plectosphaerella cucumerina BMM* (*PcBMM*). Arabidopsis plants impaired in ABA biosynthesis, such as *aba1-6* mutant, or in ABA signaling, like the *quadruple pyr/pyl* mutant, were more resistant to *PcBMM* than wild-type plants. In contrast, the *hab1-1abi1-2abi2-2* mutant impaired in three phosphatases that negatively regulate ABA signaling displayed an enhanced susceptibility phenotype to this fungus. Comparative transcriptomic analyses of *aba1-6* and wild-type plants revealed that ABA pathway negatively regulates defence genes, many of which are controlled by either the SA, JA or ET pathways. In line with these data, we found that *aba1-6* resistance to *PcBMM* was partially compromised when the SA, JA or ET pathways were disrupted in this mutant. Additionally, in the *aba1-6* plants some genes encoding cell wall-related proteins were mis-regulated. Fourier Transform InfraRed (FTIR) spectroscopy and biochemical analyses of cell walls from *aba1-6* and wild-type plants revealed significant differences in their FTIR spectratypes and uronic acid and cellulose contents. All these data suggest that ABA signaling has a complex function in Arabidopsis basal resistance, negatively regulating SA/JA/ET-mediated resistance to necrotrophic fungi.

Introduction

Plants are exposed in their natural environments to biotic and abiotic stresses. Under these conditions, plants survival depends on their ability to detect stress-associated signals and to respond to these stimuli quickly and efficiently (Bari and Jones, 2009). The mechanisms involved in regulating the activation of defensive responses upon biotic and abiotic stresses are not fully understood. Intricate networks involving different signaling pathways are required for the activation of specific plant responses to a particular stress, but how these pathways interact to balance the final output response is largely unknown (Fujita et al., 2006; Robert-Seilaniantz et al., 2007; Bari and Jones, 2009; Spoel and Dong, 2008). Plant resistance to pathogens depends on the interplay of different signaling mechanisms, such as those mediated by the hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Thomma et al., 1998; Glazebrook, 2005). In addition to these well-characterized pathways, other plant hormones, such as abscisic acid (ABA), brassinosteroids (BR), giberellins (GA) or auxins are emerging as important co-regulators of plant resistance to pathogens, including necrotrophic fungi (Bari and Jones, 2009).

ABA regulates many aspects of plant development such as seed dormancy and germination, and also controls plant response to abiotic and biotic stress (Fujita et al., 2006; Wasilewska et al., 2008). The mechanism of action of at least one arm of the ABA perception/signaling pathway has been recently characterised at the molecular level (Raghavendra et al. 2010). The activated ABA receptor was demonstrated to be a heteromeric complex formed by an ABA-binding RCAR/PYR1/PYL family member (Regulatory Component of ABA Receptor/Pyrabactin Resistance1/PYR1-Like) and a "Clade A" type 2C protein phosphatase (PP2Cs; Schweighofer et al. 2004) such as ABI1 (Abscisic acid insensitive 1), ABI2 or HAB1 (Hypersensitive to ABA 1) (Ma et al., 2009; Park et al., 2009). In the presence of ABA, the receptor blocks the phosphatase activity of PP2Cs and consequently protein kinases, such as OST1 and SnRKs, are no longer inhibited and they phosphorylate key targets of the ABA-signaling pathway (Umezawa et al., 2009; Vlad et al., 2009).

The role of ABA signaling in the regulation of plant basal resistance to pathogens is complex and not completely understood. For example, in plant resistance to necrotrophic pathogens positive and negative actions have been described for ABA depending either on the pathosystem studied, the plant developmental stage tested or the environmental conditions used for plant growth (Mauch-Mani and Mauch, 2005; Ton et al., 2009; Robert-Seilaniantz et al., 2011; García-Andrade et al., 2011). In tomato a negative regulatory role of ABA in resistance to necrotrophic pathogens, such

as the fungus *Botrytis cinerea* and the bacterium *Dickeia dadantii,* has been demonstrated. These pathogens are less virulent on the tomato *sitiens* mutant, in which ABA biosynthesis is disrupted, than in wild-type plants (Audenaert et al., 2002; Asselbergh et al., 2007; Asselbergh et al. 2008). Similarly, Arabidopsis ABA-deficient mutants (e.g. *aba2* and *aao3*) are more resistant than wild type plants to the necrotroph *B. cinerea* and to the vascular fungal pathogen *Fusarium oxysporum* (Audenaert et al., 2002; L'Haridon et al., 2011). Several molecular mechanisms were proposed to explain the enhanced resistance phenotype of Arabidopsis and tomato ABA-deficient mutants. For example, in tomato *sitiens* plants, enhanced up-regulation of defence-related transcripts (e.g. *PR-1*), accumulation of Reactive Oxygen Species (ROS), and increase in cuticle permeability were suggested to enhance resistance to *B. cinerea* and *D. dadantii* (Audenaert et al., 2002; Asselbergh et al., 2007; Curvers et al. 2010). Similarly, enhanced resistance of Arabidopsis ABA-deficient mutants (*aba2*) to *B. cinerea* was linked to increased cuticle permeability, which was also predicted to account for the stronger and faster accumulation of ROS seen following fungal infection (L'Haridon et al. 2011).

Exogenous application of ABA to Arabidopsis wild-type plants resulted in an enhanced susceptibility to *Pseudomonas syringae* pv. *tomato*, that was correlated with inhibition of lignin biosynthesis, reduced SA accumulation and suppression of transcripts of several defence-related genes, including some regulated by SA and JA/ET signaling pathways (Anderson et al., 2004; Mohr and Cahill, 2007). Treatment of Arabidopsis with ABA also blocked the activation of Systemic Acquired Resistance (SAR), both upstream and downstream of SA biosynthesis (Yasuda et al., 2008). Congruently, SAR activation disrupted the up-regulation of ABA biosynthesis and signaling in plants exposed to salt stress, further indicating a negative cross-talk between SAR and ABA-signaling pathways (Yasuda et al., 2008). Plant pathogens also utilize ABA-mediated suppression of plant defensive responses to colonize plant tissues. The AvrPtoB effector of *P. syringae* pv. *tomato* DC3000 induces the accumulation of ABA in Arabidopsis to suppress the induction of basal defence by down-regulating SA biosynthesis and signaling pathways (de Torres-Zabala et al., 2007; 2009). Further support for an important role for ABA-SA crosstalk in plant defence comes from a recent study demonstrating that activation of *EDS1/PAD4*-dependent immune responses rapidly disrupts ABA signaling transduction (Kim et al. 2011).

ABA has also a positive role in the regulation of Arabidopsis basal resistance to certain pathogens. Increased susceptibility to the necrotrophic fungus *Alternaria*

brassicicola, the vascular oomycete *Pythium irregulare* and the necrotrophic fungus *Plectosphaerella cucumerina* was observed in ABA-deficient mutants (Adie et al., 2007; Flors et al., 2008; García-Andrade et al., 2011). ABA is required for JA biosynthesis and induction of the expression of JA-regulated defence genes, two essential processes for full Arabidopsis resistance to *P. irregular* (Adie et al., 2007). ABA triggers callose deposition upon *A. brassicicola* infection, a defence mechanism associated with restriction of colonization by the fungus. A reduction of ABA levels in plants infected with *A. brassicicola* may represent a virulence defence mechanism evolved to favour colonization (Flors et al., 2008). A role for ABA in mimicking β-amino butyric acid (BABA)-primed callose deposition and some defence responses against the necrotrophic pathogens *A. brassicicola* and *P. cucumerina* has also been described (Ton and Mauch-Mani, 2004; Flors et al., 2005; García-Andrade et al., 2011). Moreover, the enhanced and constitutive accumulation of ABA of the Arabidopsis *irx1-6* mutant, impaired in a secondary cell wall cellulose synthase (AtCESA8), and of the *ocp3* mutant, defective in a transcriptional factor of the homeodomain family, has been suggested to contribute to increased resistance to several pathogens (Hernandez-Blanco et al., 2007; García-Andrade et al., 2011).

In this work we show that disruption of ABA biosynthesis or signaling leads to a constitutive activation of Arabidopsis defensive response. We provide genetic evidence that support a negative function of ABA in the regulation of SA/ET/JA-dependent immune responses, which are essential for Arabidopsis resistance to the necrotrophic fungal pathogen *P. cucumerina BMM*. Moreover, we show that *aba1-6* mutant display alterations in their cell wall structure and composition.

Results

ABA-deficient and signaling mutants are more resistant than wild-type plants to the necrotrophic fungus *P. cucumerina BMM*

To investigate the role of ABA in the regulation of Arabidopsis resistance to necrotrophic fungal pathogens, we tested the susceptibility to *P. cucumerina BMM* (*PcBMM*) of plant mutants impaired in ABA biosynthesis, like *aba1-6* (Niyogi et al., 1998), in ABA-signaling, such as the *quadr pyr/pyl* (*pyr1pyl1pyl2pyl4;* Park et al., 2009), or displaying a partial constitutive activation of ABA pathway, as it occurs in the *triple abi2-2* mutant (*hab1-1abi1-2abi2-2*; Rubio et al., 2009). We inoculated three-week old Col-0 wild-type plants and the ABA mutants with a spore suspension ($4x10^6$ spores/ml) of the *PcBMM* isolate that causes disease in a broad-range of Arabidopsis accessions

(Llorente et al., 2005; Sánchez-Vallet et al., 2010). Progression of the infection was examined at different hours/days post inoculation (hpi/dpi) by determining the fungal growth and plant cell death, using trypan blue (TB) staining of inoculated leaves, the fungal biomass by quantitative PCR (qPCR) of *PcBMM β-Tubulin* gene, and by macroscopic evaluation of disease ratings (DR) (Sánchez-Vallet et al., 2010). As shown in Figure 1, *aba1*-6 and *quadr pyr/pyl* mutants supported lower fungal growth than wild-type plants as revealed by TB staining of inoculated leaves 1 dpi and by fungal biomass quantification 3 dpi (Figure 1A, C). In contrast, fungal growth in the *triple abi2-2* mutant was higher than that determined in Col-0 plants (Figure 1A, C). These results were corroborated by confocal microscopy analyses of Col-0 and *aba1-6* plants inoculated with *PcBMM-GFP,* a fungal transformant constitutively expressing the Green Fluorescence Protein (GFP; Supplemental Figure 1; Ramos et al. 2012). A positive correlation was found between *in planta* fungal biomass, plant cell death and macroscopic disease symptoms (Figure 1A, B, D and data not shown). Thus, the level of plant cell death, as determined by TB staining, was lower in *aba1*-6 and *quadr pyr/pyl* mutants than in wild-type plants, whereas it was enhanced in the triple *abi2-2* mutant (Figure 1D). Accordingly, the *aba1*-6 mutant, that has a dwarf phenotype (Barrero et al., 2005), and the *quadr pyr/pyl* mutant displayed similar, less severe macroscopic symptoms than those observed in inoculated wild-type plants, whereas the opposite was true for the *triple abi2-2* mutant (Figure 1B and data not shown). These data strongly supported a negative function of the cytosolic ABA perception and signaling pathways in the regulation of Arabidopsis resistance to *PcBMM* and further corroborated the relevant function of ABA pathway in the control of Arabidopsis resistance to necrotrophic fungal pathogens

ROS regulates Arabidopsis response to biotic and abiotic stresses (Torres et al., 2005). ABA has been suggested to negatively regulate ROS accumulation in Arabidopsis and tomato in resistance responses to the necrotrophic fungus *B. cinerea* (Asselbergh et al., 2007; Curvers et al., 2010; L'Haridon et al., 2011). Therefore, we used 3-3'-diaminobenzidine (DAB) staining to determine ROS production in *PcBMM*-inoculated wild-type plants and in the three ABA-mutants, but no significant differences were macroscopically observed in ROS production among the genotypes tested (Figure 1E), further indicating that ABA-mediated regulation of Arabidopsis resistance to this fungus is independent of ROS production. ABA has also been described to trigger callose deposition upon pathogen infection and ABA-deficient mutants were shown to be impaired in callose accumulation after inoculation with some fungal pathogens (Ton and Mauch-Mani, 2004; Garcia-Andrade et al., 2011). However,

PcBMM infected plants stained with Aniline Blue displayed lower callose content in the *aba1-6* and *quadruple pyr/pyl* mutants than in wild-type plants (Figure 1F, G), indicating that callose deposition is not determining the enhanced resistance phenotype of these mutants. In line with these results we found that the *pmr4* mutant, impaired in callose deposition (Nishimura et al., 2003), was as susceptible to *PcBMM* as wild-type plants (Supplemental Figure 2). This *pmr4* phenotype was not due to the previously described enhanced SA content of this mutant (Nishimura et al., 2003), as the susceptibility to *PcBMM* of the *pmr4 sid2-1* double mutant, defective in SA biosynthesis, was similar to that of susceptible *sid2-1* plants (Supplemental Figure 2). These results further corroborate that, under our inoculation conditions, callose deposition is not an essential defensive barrier against the necrotrophic fungus *PcBMM*, and that additional defence mechanisms determine the enhanced resistance phenotype of *aba1-6* and *quadruple pyr/pyl* mutants.

Comparative transcriptomic analyses reveal constitutive activation of a complex defensive network in the *aba1-6* mutant

To further characterise the molecular mechanisms regulating ABA-mediated resistance to necrotrophs, we selected the well-characterised *aba1-6* mutant (Niyogi et al., 1998; Barrero et al., 2005) and we performed a comparative transcriptomic analysis of Col-0 and *aba1*-6 plants prior inoculation (t=0) and at 1 day after water treatment (Mock, M) or inoculation with a spore suspension ($4x10^6$ spores/ml) of *PcBMM*. Out of the 22,000 probesets tested in these transcriptomic analyses, 8,687 showed statistically significant differential abundance according to a two-way analysis of variance and a Benjamini and Hochberg multiple testing correction ($P \leq 0.01$; Gene-Spring 7.2 software). From these probesets, 213 were differentially regulated in untreated (t=0) *aba1-6* compared to untreated wild-type plants (Supplemental Table 1). In *PcBMM*-inoculated *aba1-6* plants 497 probesets were differentially expressed compared to mock-treated plants whereas 1,474 probesets were found differentially expressed between *PcBMM* and mock inoculated wild-type plants (Supplemental Tables 2 and 3).

The 213 probesets differentially regulated in the untreated (t=0) *aba1-6* plants (Supplemental Table 1) were analysed by hierarchical clustering. As shown in Figure 2A, a significant overlap was found between the transcriptional profile of untreated *aba1-6* and of *PcBMM*-inoculated wild-type plants. In agreement, 128 from the 213 probesets (60%) that were constitutively up- or down-regulated in untreated *aba1-6* plants were found to be also differentially regulated in wild-type plants upon *PcBMM*-inoculation (Figure 2B; Supplemental Table 4). Thus, 67% (90 genes) of the genes

constitutively up-regulated (134 genes) in *aba1-6* mutant plants were also induced in wild-type plants upon *PcBMM* infection (Figure 2B) and might be considered as constitutively up-regulated defensive genes. Meta-analysis, using Genevestigator tools (Zimmermann et al., 2004), demonstrated that the expression of a high number of these up-regulated genes were also induced after Arabidopsis infection with other necrotrophic fungi such as *B. cinerea* (60 genes), and with other pathogens like *Phytophthora infestans* (70 genes)*, Erysiphe orontii* (27 genes), *E. cichoracearum* (31 genes) or *P. syringae* (19 genes); in contrast, a reduced number of these genes were found to be differentially regulated after insect or nematode attack (8 and 5 genes, respectively). About 63% (57 genes) of the genes up-regulated both in *aba1-6* untreated plants and in *PcBMM*-inoculated wild-type plants were also up-regulated by either JA, SA or ET treatment. These data indicated that an extensive crosstalk existed among these pathways and ABA signaling.

In addition, 33 (42%) out of the 79 probesets down-regulated in untreated *aba1-6* were also down-regulated after infection of wild-type plants with *PcBMM*. Interestingly, expression of a high percentage of these genes was also down-regulated after infection with *B. cinerea* (67%) and *P. infestans* (48%)*,* as revealed by Meta-analysis (data not shown). The expression of 30% of these *aba1-6* and *PcBMM* down-regulated genes was found to be repressed either by SA or ET treatments, further supporting the existence of a negative interplay among these signaling pathways in response to pathogen infection.

Functional classification of the proteins encoded by the genes differentially regulated in untreated *aba1-6* mutant and in *PcBMM*-inoculated wild-type plants was performed using Classification SuperViewer tool (BAR, Bio-Array Resource; http://bar.utoronto.ca/; Toufighi et al., 2005). Notably, the most over-represented cellular component categories were “cell wall” (p-value=$7x10^{-15}$) and “extracellular” (p-value=$3x10^{-9}$), and the most over-represented biological process categories were “response to stress” (p-value=$2x10^{-17}$) and “response to abiotic and biotic stimulus” (p-value=$2x10^{-12}$; Figure 2C). Among the proteins in the “biotic stress” functional category there were some previously described to be involved in defence against necrotrophs (Table 1): i) PDF1.2, PR-1, and PR-4, defensive markers of the JA/ET, SA and ET pathways, respectively; ii) the Respiratory burst oxidase homolog D (RbohD), an enzymatic subunit of the plant NADPH oxidase involved in the generation of H_2O_2; iii) the transcription factors WRKY33 and ORA59; and iv) several proteins, such as CYP81F2, PAD3 and PEN3, involved in the synthesis and delivery of Tryptophan (Trp)-derived secondary metabolites, like camalexin and indole glucosinolates (IGs).

Similarly, some genes encoding cell wall-related proteins were found to be mis-regulated in *aba1-6* mutant compared with wild-type plants (Supplemental Table 5). Remarkably, cell wall architecture/alteration has been recently linked to the regulation of tomato and Arabidopsis resistance to necrotrophic pathogens, such *B. cinerea* and *PcBMM*, respectively (Asselbergh et al., 2007; Sánchez-Rodríguez et al., 2009; Delgado-Cerezo et al., 2012).

To validate the transcriptomic data, the steady-state expression of some defensive genes (*PDF1.2*, *PR-1*, *PR-4* and *ORA59*), which were up-regulated in *aba1-6* plants, was determined in untreated wild-type plants and the ABA resistant mutants (*aba1*-6 and *quadr pyr/pyl*). qPCR analysis demonstrated that the expression of these genes was higher in both ABA-deficient and ABA-signaling mutants than in wild-type plants (Figure 2D). The role of one of these differentially expressed genes in Arabidopsis resistance to *PcBMM* was validated by demonstrating the enhanced susceptibility to this fungus of *wrky33-1* mutant, which is defective in the WRKY33 transcriptional factor previously shown to control the resistance to the necrotroph *B. cinerea* (Supplemental Figure 3; Zheng et al., 2006). These data further corroborated the relevance in plant immunity of constitutively up-regulated genes in the Arabidopsis *aba1*-6 and *quadr pyr/pyl* mutants.

Hormonal regulation of the genes differentially expressed in *aba1*-6 plants

To gather additional information on the processes regulated by genes differentially regulated in *aba1-6*, meta-analysis was performed of the transcriptomic data of untreated *aba1-6* mutant (Supplemental Table 1), wild-type plants inoculated with *PcBMM* (Supplemental Table 2), and publicly available transcriptomic data of Arabidopsis wild-type plants treated with the hormones SA, ACC (1-aminocyclopropane-1-carboxylic-acid, an ET precursor) or methyl jasmonate (MJ) (Goda et al. 2008). Using Pearson Uncentered Hierarchical Clustering on genes differentially regulated in *aba1-6* compared to wild-type plants upon inoculation with *PcBMM*, we found out that the transcriptomic profiles of untreated *aba1-6* mutant and of *PcBMM*-inoculated wild-type plants clustered together and separately from the transcriptomic profiles of plants treated with the different hormones (Figure 3), indicating that the overall transcriptomic profile of *aba1-6* mutant could not be linked specifically to any of these hormones. Pearson Uncentered Hierarchical Clustering differentiated seven statistically significant gene clusters using a distance threshold of 0.7 (Figure 3 and data not shown). The largest clusters (I through IV) were further

analysed: Clusters I-III corresponded to genes up-regulated in both untreated *aba1-6* and *PcBMM*-inoculated wild-type plants, whereas cluster IV contained genes down-regulated in both *PcBMM*-inoculated wild-type plants and untreated *aba1-6* plants (Supplemental Table 6).

The presence of conserved putative *cis*-regulatory elements was determined in the genes included in clusters I-IV with Transplanta software (http://bioinfogp.cnb.csic.es/transplanta_dev/). Subsequently, TOMTOM (http://meme.sdsc.edu/meme/cgi-bin/tomtom.cgi; Gupta et al 2007) and PLACE databases (Plant Cis-acting Regulatory DNA Elements Database; http://www.dna.affrc.go.jp/PLACE/; Higo et al 1999) were used to identify the statistically overrepresented regulatory elements in the promoters of the genes from each cluster. We observed that cluster I contained genes (14 out of 23) predicted to be predominately up-regulated by JA-treatment, some of which encoded proteins (RbohD, PEN3, ATKTI1 and ATTI1) involved in plant basal resistance (Supplemental Table 6). Notably, the *cis*-regulatory elements of these JA-dependent genes were enriched in W-BOX sequences, suggesting a transcriptional regulation by WRKY transcription factors (Rushton et al., 1996). Cluster II contained genes (18 out of 19) associated with SA-mediated induction, such as defence-related genes like *PR-1, WRKY33, PNP-A* (encoding the plant natriuretic peptide A), *ATGRXS13* and a *R*eceptor *L*ike *K*inase (*RLK*, Table 1). Among these genes there were no *cis*-regulatory elements statistically over-represented. The 44 genes in Cluster III, that were not predominantly regulated by any of the hormones analysed, encoded proteins involved in biotic and abiotic stress responses, such as CHIB, PR-4, PRX33 and PRX34 (Table 1), cell wall-related proteins, and two transcription factors (WRKY26 and ORA59). The W-box *cis*-regulatory element was also over-represented in the promoters of Cluster III genes. Finally, nearly all the genes down-regulated in untreated *aba1-6* plants were contained in cluster IV and they were found to be negatively regulated by either ACC or SA treatment and, to a lesser extent, by JA-signaling pathway. Among the genes found in this cluster several encoded cell wall-related proteins (Figure 3 and Supplemental Table 6). There were no *cis*-regulatory elements statistically over-represented in cluster IV genes.

The cluster analyses suggested that a significant number of the genes differentially regulated in *aba1*-6 plants responded to different hormones, in particular to SA and JA. Moreover, a significant number of the differentially expressed genes in *PcBMM*-inoculated wild-type plants corresponded to genes that are up-regulated (110 probesets) or down-regulated (87 probsets) by ABA. Accordingly, we measured the

concentration of these hormones in ABA-deficient and signaling mutants and in wild-type plants to evaluate if the mis-regulation of these genes could be linked to alterations in hormone levels. SA content was not altered in untreated *aba1-6* and *quadr pyr/pyl*, while it was lower in the *triple abi2-2* mutant plants (Supplemental Figure 4A). The concentration of JA was lower in the three mutants than in wild-type plants (Supplemental Figure 4A). As expected, *aba1-6* mutant displayed lower ABA content than wild-type plants and *quadr pyr/pyl* and *triple abi2-2* mutants, that are not impaired in ABA biosynthesis (Supplemental Figure 4A). Inoculation of wild-type plants with *PcBMM* (1 dpi) induced an increase in the concentration of JA and SA (Supplemental Figure 4B), which was consistent with the previously described positive role of SA and JA in the regulation of Arabidopsis resistance to this fungal pathogen (Berrocal-Lobo et al., 2002). Interestingly, the elevation of JA and SA content in the *PcBMM*-inoculated ABA-mutants did not exceed that in wild-type plants (Supplemental Figure 4B). Notably, significant differences in the levels of ABA were not found among *PcBMM*-inoculated and mock-treated wild-type plants (Supplemental Figure 4B).

SA, JA and ET signaling pathways are essential for *aba1*-mediated resistance to *PcBMM*

To further determine if the enhanced resistance of *aba1-6* plants to *PcBMM* was dependent on JA, SA and ET signaling, we genetically disrupted these pathways in *aba1-6* mutant by generating the *sid2-1 aba1-6*, *ein2-1 aba1-6*, *aos aba1*-6 and *coi1-1 aba1*-6 double mutants (Figure 4 and data not shown), which were impaired, respectively, in the SA biosynthesis, the ET-signaling and in the JA biosynthesis and signaling. All these double mutants were viable and showed developmental phenotypes that in some cases were a combination of those observed in their corresponding parental lines (Supplemental Figure 5). Susceptibility of these double mutants to *PcBMM* was analysed by spraying them alongside their parental lines and wild-type plants, with a spore suspension ($4x10^6$ spores/ml) of the fungus. Notably, fungal biomass and DR were significantly higher in *sid2-1 aba1-6*, *ein2-1 aba1-6*, *aos aba1-6* and *coi1-1 aba1-6* double mutants than in *aba1-6* single mutant (Figure 4A, B and data not shown), indicating that the SA, ET and JA signaling pathways are essential for the enhanced resistance of *aba1-6* to the necrotrophic fungus *PcBMM*. However, none of the double mutants tested was as susceptible as their corresponding parental lines *sid2-1*, *ein2-1* or *aos* (Figure 4A, B). These data indicate that all these

signaling pathways partially contributed to *aba1*-mediated resistance, as any of them on its own was sufficient to explain the enhanced resistance phenotype of *aba1-6*.

The impact of hormone imbalance in the resistance phenotype observed in the *sid2-1 aba1-6*, *ein2-1 aba1-6* and *aos aba1-6* double mutants was studied by quantifying the expression levels of *PR-1, PR-4, LOX2* and *PDF1.2* (marker genes of the SA, ET, JA and ET/JA signaling, respectively). The steady-state expression of *PR-1*, which was higher in the *aba1-6* mutant than in the wild-type plants (Figure 2D), was further enhanced in the *ein2-1 aba1-6* and *aos aba1-6* double mutants (Figure 4C). As expected, *PR-1* expression was not detected in the *sid2-1* and *sid2-1 aba1*-6 mutants impaired in SA biosynthesis (Figure 4C). These data suggested a negative regulation of *PR-1* expression by ABA, ET and JA signaling pathways. The enhanced expression of *PR-4* in the *aba1-6* mutant was disrupted in the *ein2-1 aba1*-6 plants, but not in the *sid2-1 aba1-6* and *aos aba1-6* double mutants, further corroborating the ET-dependent regulation of *PR-4* gene in the *aba1-6* background (Figure 4C). No significant changes were observed in the expression levels of *LOX2*, a marker gene for the JA-signaling pathways, in all the genotypes tested (Figure 4C). The enhanced expression of *PDF1.2* gene in the *aba1-6* mutant (Figure 2D and Figure 4C), was partially blocked in the *sid2-1 aba1-6* and *ein2-1 aba1-6* double mutants, whereas it was not affected in the *aos aba1-6* genotype (Figure 4C). All together these data illustrate the complex interplay between hormone signaling pathways in the regulation of defensive genes in Arabidopsis resistance responses.

JA, SA and ET signaling pathways do not interfere with ABA function in the regulation of Arabidopsis resistance to abiotic stresses

ABA signaling has also a role in the regulation of plant responses to abiotic stress, such as drought and osmotic stress. Thus, the *aba1* mutant was shown to be impaired in ABA-mediated resistance to desiccation (Xiong et al. 2002). Taking advantage of the double mutants generated in this work, we decided to assess whether the SA, ET and JA-signaling pathways might interfere with the regulatory function of ABA signaling in abiotic stresses. We determined plant water loss (represented as % loss weight) at different time points (0 to 3 hours) in wild-type plants and the single and double mutants. As shown in Figure 5, water loss was similar in Col-0, *sid2-1*, *ein2-1* and *aos* genotypes, which harboured the wild-type *ABA1* allele. Interestingly, the *sid2-1 aba1-6*, *ein2-1 aba1-6* and *aos aba1-6* double mutants were as sensitive to this abiotic stress as the single mutant *aba1-6*, which showed an enhanced and faster water loss than

that determined in Col-0 plants (Figure 5). These data indicated that ET, SA and JA-signaling did not play a major role in the regulation of Arabidopsis responses to this abiotic stress and that they did not interfere with ABA function in the regulation of this process.

ABA modulates Arabidopsis cell wall composition

Arabidopsis resistance to necrotrophic fungi, such as *PcBMM*, can be regulated by remodelling plant cell wall structure and composition (Hernández-Blanco et al., 2007; Cantu et al., 2008; Sánchez-Rodríguez et al., 2009; Ramírez et al., 2011; Delgado-Cerezo et al. 2012). Ontology analyses of the genes differentially regulated in untreated *aba1-6* plants identified cell wall-related genes mis-regulated in the mutant compared with wild-type plants (p-value $7x10^{-15}$; Supplemental Table 5). Thus, we determined cell wall structure/composition of the *aba1-6* mutant and compared it with that of wild-type plants. Cell walls from non-inoculated leaves of three-week old *aba1-6* and Col-0 plants were subjected to Fourier Transform InfraRed (FTIR) spectroscopy to obtain qualitative spectratypes (i.e., cell wall phenotypes). The comparison of averaged FTIR spectra (n=15) obtained between *aba1-6* and Col-0 showed significant differences (Figure 6A). Much of the total sample variation (86%) was explained by principal component 1 (PC1; Figure 6B). Wavenumbers at 1056 cm^{-1}, 1080 cm^{-1} and 1109 cm^{-1}, that are common in the fingerprint for several cell-wall polysaccharides (Kauráková et al. 2000), and 1544 cm^{-1} and 1654 cm^{-1}, that are associated to proteins, were relatively more abundant in Col-0 than in *aba1*-6 cell walls (Figure 6C). On the contrary, bands at 825 cm^{-1}, 889 cm^{-1} and 935 cm^{-1} were more intense in *aba1*-6 than in wild-type plants (Figure 6C). Due to the complexity of IR spectra even after PCA analysis was difficult to assign IR bands differences to specific cell wall polysaccharides.

To obtain a more quantitative analysis of the cell wall changes in *aba1*-6 plants we determined the non-cellulosic neutral monosaccharide composition of leaves from non-inoculated three-week old *aba1-6* and Col-0 plants (Blakeney et al., 1983; Reiter et al., 1993) as well as cellulose and uronic acid content of their walls (Figs 6D-E). We found that the amount of cellulose was much lower in *aba1-6* samples than in wild-type plants whereas the content of uronic acid was much higher in the mutant than in the wild-type plants (Figure 6D). No significant changes were observed in non-cellulosic neutral sugar between *aba1-6* samples and wild-type plants (Figure 6E). To further correlate these cell wall alterations in *aba1-6* mutant with its resistance phenotype, we

determined the cellulose and uronic acid content, as well as the non-cellulosic neutral monosaccharide composition in leaves from non-inoculated three-week old *quadr pyr/pyl* and *triple abi2-2* mutants. As showed in Supplemental Figure 6, not significant alterations in the content of uronic acid and cellulose were found between these mutants and wild-type plants, whereas a subtle reduction in the content of non-cellulosic neutral sugars was found in the *triple abi2-2* compared with that in wild-type plants. All together, these results revealed that disruption of the ABA biosynthesis, but not ABA signaling, leads to a modification of cell wall structure and composition. However the lack of correlation between altered cell wall chemistry and basal resistance in the *pyr/pyl* mutant and the triple *abi2-1* mutant suggests that these cell wall modifications have no significant contribution to ABA-regulated resistance

Discussion

ABA has multiple and diverse functions in plants, such as the regulation of developmental-associated processes, the response to abiotic stresses and the outcome of pathogen infections (Fujita et al., 2006; Wasilewska et al., 2008). For the latter, positive and negative functions have been described for ABA depending on the specific plant-pathogen interaction studied (Mauch-Mani and Mauch, 2005; Ton et al., 2009). Here, we show that ABA plays a negative regulatory function in balancing Arabidopsis resistance response to *PcBMM*, a necrotrophic fungal pathogen that naturally colonizes a broad-range of Arabidopsis accessions (Llorente et al., 2005; Sánchez-Vallet et al., 2011). The *aba1-6* mutant defective in ABA biosynthesis and the *quadr pyr/pyl* mutant impaired in four ABA-receptors (Niyogi et al., 1998; Park et al., 2009) were more resistant to *PcBMM* than wild-type plants, whereas the triple *hab1-1abi1-2abi2-1* mutant, that has a constitutive activation of ABA-signaling due to the impairment of three PP2C phosphatases (from clade A) that negatively regulate ABA signaling (Rubio et al., 2009), displayed an enhanced susceptibility to this fungal pathogen (Figure 1). Thus, in addition to ABA biosynthesis, we show here that ABA signaling pathway is essential for suppression of Arabidopsis resistance to necrotrophic fungi, as impairment or constitutive activation of the pathway lead to enhanced or reduced resistance responses, respectively. In line with these data, treatment of the ABA-deficient mutant *aba1-1 (*L*er* background) with ABA (50 μM) reverted its enhanced resistance to *PcBMM* to the susceptibility levels observed in wild-type plants (Supplemental Table 7). The data presented here are consistent with the negative regulatory role of ABA seen on resistance in tomato to the necrotrophic pathogens *B.*

cinerea and *D. dadantii*, and in Arabidopsis to the fungi *F. oxysporum* and *B. cinerea* (Audenaert et al., 2002; Anderson et al., 2004; Asselbergh et al., 2008; L'Haridon et al., 2011). In contrast, ABA plays a positive regulatory role in Arabidopsis basal resistance to the necrotrophic fungus A. *brassicicola* or the vascular oomycete *P. irregular*, and in the modulation of the *ocp3* resistance to necrotrophic fungi (Adie et al., 2007; Flors et al., 2008; García-Andrade et al., 2011). Strikingly, a positive role of ABA in resistance to *P. cucumerina* has been previously described (e.g. *aba2* mutant was shown to be hypersusceptible to the fungus), and this function has been associated to the regulation of callose deposition upon infection (García-Andrade et al., 2011). However, in these experiments the plants used for inoculation were older (five-week old) than those used here (three-week old) and drop-inoculation instead of spray-inoculation of expanded leaves was performed (García-Andrade et al., 2011). Moreover, under our experimental conditions callose deposition does not seem to be essential for resistance to *PcBMM* as the susceptibility of the callose-deficient *pmr4* plants to the fungus was similar to that of wild-type plants (Supplemental Figure 2). The data presented here reinforce the different roles that ABA plays in plant basal resistance depending on the plant/pathogen interaction analysed, and also suggest a putative effect of plant developmental stage and growth environmental conditions on ABA regulatory function. This last conclusion is in line with the recently described effect of humidity on the effectiveness of Arabidopsis wound-induced resistance to *B. cinerea* (L´Haridon et al., 2011).

Comparative transcriptomic analysis of the *aba1-6* mutant and wild-type plants demonstrated that in *aba1-6* there is a constitutive activation of resistance responses as the expression of a significant subset of defence-related genes was up-regulated in the mutant compared with wild-type plants (Table 1). Furthermore, we found a significant overlap (60% of the genes) between the transcriptomic profiles of untreated *aba1-6* mutant and *P. cucumerina*-infected wild-type plants 1 dpi. This is a clear indication that the Arabidopsis defensive responses activated upon fungal infection were constitutively up-regulated in *aba1-6* plants, and therefore that ABA negatively regulates these responses against this fungal pathogen. Among the defensive proteins encoded by constitutively up-regulated genes in *aba1-6* were antimicrobial proteins (e.g. PDF1.2, PR-1 and LTP3), enzymes involved in the biosynthesis and target delivery of tryptophan derived secondary metabolites, such as IGs and camalexin (e.g PEN3, PAD3 and CYP81F2), and the ATRBOHD and PRX33/PRX34 proteins that are involved in ROS generation (Figure 2). The enhanced accumulation of antimicrobial proteins and secondary metabolites in Arabidopsis has been shown to confer broad-

spectrum resistance to pathogens, including the necrotroph *PcBMM* (Garcia-Olmedo et al., 2001; Stein et al., 2006; Hernández-Blanco et al., 2007; Bednarek et al., 2009; Sánchez-Vallet et al., 2010). ROS accumulation has been linked to the enhanced resistance phenotype of some ABA mutants (Asselbergh et al., 2007; L'Haridon et al. 2011), but a significant increase in ROS production upon *PcBMM* infection was not observed in the ABA mutants in comparison to wild-type plant (Figure1E). Notably, some transcriptional factors, such as WRKY33 and ORA59, that regulate Arabidopsis immune responses to pathogens, including necrotrophic fungi (Supplemental Figure 3; Zheng et al., 2006; Pré et al., 2008), were also up-regulated in unchallenged *aba1-6* and *quadr pyr/pyl* mutants (Figure 2 and data not shown). Interestingly, the *cis*-regulatory elements of the *aba1-6* up-regulated genes were enriched in W-box and GCC-box sequences that bind transcriptional factors such as WRKY33 and ORA59, respectively (Rushton et al., 1996; Zarei et al., 2011). These data further corroborate the link between ABA-signaling and the Arabidopsis resistance response to the necrotrophic fungus *PcBMM.*

Hierarchical clustering of genes differentially regulated in *aba1-6* mutant identified four statistically significant clusters (I-IV; Figure 3 and Supplemental Table 6). The function of the genes contained in these cluster suggested an extensive and complex crosstalk among different signaling pathways. For example, two of the *aba1-6* clusters contained up-regulated genes whose expression was positively modulated by either JA or SA treatments (Figure 3). Genetic disruption of these pathways by generating the *coi1-1 aba1-6* and *aos aba1-6,* and *sid2-1aba1-6* double mutants led to a partial impairment of *aba1-6* resistance phenotype (Figure 4 and data not shown). Similarly, the cluster analysis suggested an ET function in *aba1-6* resistance, which was further corroborated by showing that the *ein2-1 aba1-6* double mutant was more susceptible to *PcBMM* than *aba1-6* plants (Figure 4). These data are in accordance with previous reports describing the antagonistic interaction between ABA and the JA, ET or SA signaling pathways in the regulation of plant resistance to pathogens. With a few exceptions, these antagonistic interactions were hypothesized based on comparative gene expression analysis (Anderson et al., 2004; Adie et al., 2007; de Torres-Zabala et al., 2007; Asselbergh et al., 2008; Flors et al., 2008). Here, we have genetically validated some of the interactions of this complex network and also have found additional, previously uncharacterised ones (Figure 7). Notably, the interaction between ABA and the JA and SA hormones in balancing the resistance response of *aba1-6* to *PcBMM* seems to take place at the signaling level as the content of these hormones in *aba1-6* and *quadr pyr/pyl* mutants (untreated or *PcBMM*-inoculated) was

not higher than those found in wild-type plants. Moreover, ABA content was not increased in *PcBMM*-inoculated wild-type plants (Supplemental Figure 4) and exogenous application of ABA (50 μM) did not have any effect on the resistance of wild-type plants, whereas it restored the resistance of *aba1* mutant to the susceptible levels of wild-type plants (Supplemental Table 7). In contrast to their genetic interaction in the regulation of Arabidopsis innate immunity, the JA, ET and SA signaling do not interfere with ABA function in the regulation of Arabidopsis response to abiotic stress, such as drought (Figure 5).

The intermediate resistance phenotype observed in the double mutants in comparison to that of *aba1-6* plants suggest that ABA, JA, ET and SA signaling pathways cooperatively contribute to Arabidopsis resistance to *PcBMM*. How these pathways interact at the molecular level to regulate this resistance needs further characterization, but seems to be more complex than initially anticipated (Tsuda et al., 2009). For example, the constitutive overexpression of the SA-marker gene *PR-1* in the *ein2-1 aba1-6* and *aos aba1-6* double mutants (Figure 4C) was not linked to an enhanced accumulation of SA in these double mutants (data not shown). Moreover, the ET/JA marker gene *PDF1.2* was similarly up-regulated in the *aba1-6* plants and in the *aos aba1-6* double mutant defective in JA biosynthesis (Figure 4C). Therefore, the results presented here indicate that an intricate and complex, regulatory network among these pathways occurs upon Arabidopsis challenge with necrotrophic pathogens (Figure 7). It cannot be discarded that additional hormones such as auxin, giberellin, cytokinin and brassinosteroids might be involved in this multifaceted regulatory network conferring resistance to necrotrophic fungi (Bari and Jones, 2009).

Comparative transcriptomic analyses and genetic data revealed that in addition to the defensive responses regulated by hormones, other mechanisms might contribute to explain the resistance phenotypes of ABA mutants. For example, a significant number of the *aba1-6* differently regulated genes encoded proteins that were related with cell wall biosynthesis/modification (Figure 2C). In line with these data we found that *aba1-6* plants, which have a dwarf phenotype, showed alterations in the structure/composition of their walls compared with that of wild-type plants (Figure 6), further suggesting that *aba1-6* resistance can be partially dependent on these wall alteration. Our data are in line with the described cell wall alterations found in the tomato ABA-deficient *sitiens* mutant (Asselbergh et al., 2008) and with the hypothesized modulation of cell wall architecture by ABA signaling (Gimeno-Gilles et al., 2009). The relevance of cell wall in directly mediating the resistance of Arabidopsis to different pathogens, including *PcBMM* has been reported previously (Hernandez-

Blanco et al., 2007; Denoux et al., 2008; Sánchez-Rodríguez et al., 2009; Ramirez et al., 2011; Delgado-Cerezo et al., 2012). However, we did not find similar wall modifications in the *PcBMM*-resistant *quadr pyr/pyl* mutant and therefore it is tentative to speculate about the putative contribution of *aba1-6* wall alterations to *PcBMM* resistance. Also, a decrease in cuticle permeability and the alteration in callose deposition at the cell wall have been suggested to explain the resistance phenotypes of Arabidopsis and tomato ABA-deficient mutants (Ton et al., 2004; Asselbergh et al., 2008; Garcia-Andrade et al., 2011; L'Haridon et al., 2011). However, in our plant growth and inoculation conditions callose does not seem to contribute to the enhanced resistance to *PcBMM* of the *aba1-6* and *quadr pyr/pyl* mutants (Figure 1E; Supplemental Figure 2). In conclusion, with this work we contribute to elucidate the complex role that phytohormones, in particular ABA, play in balancing plant resistance to pathogens.

Material and Methods

Biological material and growth conditions

Arabidopsis thaliana plants were grown in phytochambers on a sterilized mixture of soil and vermiculite (3:1) with a 10 h day/14 h night photoperiod, a temperature of 22ºC day/20ºC night and 50% relative humidity (Hernández-Blanco et al., 2007). Light intensity was fixed to 120–150 µmol m^{-2} s^{-1}, according to Weigel and Glazebrook (2002). The following lines, in the Col-0 background, were used: *aba1-6* (Niyogi et al., 1998), *pyr1pyl1pyl2pyl4* (Park et al., 2009), *hab1-1abi1-2abi2-2* (Rubio et al, 1999), *sid2-1* (Nawrath and Metraux, 1999), *ein2-1* (Guzman and Ecker, 1990), *agb1-1* (Lease et al., 2001), *coi1-1* (Xie et al., 1998), *wrky33* (*salk_006603*), and *pmr4* and *pmr4 sid2-1* (Nishimura et al., 2003. The *aos* mutant is in Col-6 (*gl1*; Park et al., 2002) and the *aba1-1* allele is in L*er* background (Koornneef et al., 1982). The *Plectosphaerella cucumerina BMM* isolate was provided by Dr B. Mauch-Mani (University of Neuchatel, Switzerland).

Double mutants were generated by standard genetic crosses followed by identification of the mutant alleles. Genotyping of the *aba1-6* mutation was confirmed by sequencing the PCR product of 5'-GAATCGGAGGATTGGTGTTTG- 3' and 5'-CTCCAGAATCTTCAAAATCAAC-3'. The *aos* mutation was confirmed by detection of

the T-DNA insert using the flanking primers (5'-GTACGATCAAAGCCGTTGAAATTCCGAGTTTT-3' and 5'-TGGAACAAGAAAACAGATGGACTACACAGGT-3' and the T-DNA internal primer (5'-CGGGCCTAACTTTTGGTGTGATGATGCT-3'; Park et al., 2002). *sid2-1* mutation was detected by selective digestion with MunI of the product of 5'-TGTCTGCAGTGAAGCTTTGG-3' and 5'-CACAAACAGCTGGAGTTGGA-3'. The *ein2-1* mutation was selected as reported (Guzman and Ecker, 1990). The *coi1-1* mutant was selected by determining plant insensitivity to JA (Xie et al., 1998).

Biological assays

Three-week-old Arabidopsis plants were spray-inoculated with a spore suspension (4 x 10^6 spores/ml) of *PcBMM* (0.6 ml per pot containing 5 plants). After inoculation plant were transferred to a growth chamber with a 10 h day/14 h night photoperiod, a temperature of 24ºC day/22ºC night, and light intensity was maintained at 120–150 µmol m^{-2} s^{-1}. Inoculated plants were kept under high humidity in covered traits. Disease progression was estimated by determining the average Disease Rating (DR, 0–5; 0, no infection; 5, dead plant), trypan blue staining and relative quantification of fungal DNA by qPCR as previously described (Sánchez-Vallet et al., 2010). At least three independent experiments were performed, and statistically significant differences among the inoculated Arabidopsis genotypes were determined by one-way analysis of variance and Bonferroni post hoc test, as previously reported (Sánchez-Rodríguez et al., 2009). For ABA treatment, *aba1-1* and wild-type plants were sprayed with ABA (50 µm) 24 and 48 hours prior inoculation with *PcBMM* and then the DR was determined at different dpi and expressed as percentage of disease susceptibility in wild-type plants (100%). Water loss assays were performed as described by Saez et al. (2006). Three samples containing four rosettes of three-week old plants were excised and weighed separately at different time points and the average values (± SE) were determined. This experiment was performed three times.

Plant tissue staining

Trypan Blue staining was performed on mock and *PcBMM*-inoculated leaves at 20 hpi (for hyphal growth analysis) and 48 hpi (for cell death detection) (Sánchez-Vallet et al. 2010). To determine the production of H_2O_2, 3',3'-diaminobenzidine (DAB; Sigma, USA) staining of at least 6 leaves were collected 24 hpi and vacuum-infiltrated with the DAB solution (1mg/ml), placed in a plastic box under high humidity until brown precipitate was observed (3 hours) and then fixed with 3:1:1 ethanol:lactic acid:glycerol (Torres et

al., 2002). Callose deposition was determined by Aniline Blue staining in leaves harvested 24 hpi, which were incubated in 95% of ethanol and then washed with a solution of 0.07 M phosphate buffer (*p*H 7), prior to their staining with Aniline Blue (0.02%, in 0.07 M phosphate buffer, *p*H 7) (Luna et al., 2011). Leaves were mounted on slides with glycerol and observed with epifluorescence microscope using UV filter. Pixel quantification of callose was performed using Adobe Photoshop (Luna et al., 2011). At least four leaves per genotype were used for pixel quantification in the experiments performed.

Gene expression analyses

RNA extractions from *PcBMM*–inoculated or mock-treated plants were done as described (Llorente et al., 2005). Real-time qPCR analyses were preformed as previously reported (Sánchez-Vallet et al., 2010). The oligonucleotide sequences, designed using PRIMER EXPRESS v2.0 (Applied Biosystems), used for qPCR have been described previously (Sánchez-Vallet et al., 2010), except those of *ORA59* (5'-GCAGCCTCGCAGTACTCAATTT-3' and 5'- TCAAGGCTATCACCGGAGACTC-3') and *LOX2* genes (5'-ATCAACAAGCCCCAATGGAA-3' and 5'-CGGCGTCATGAGAGATAGCAT-3'). qPCR results are mean values (±SDs) from two technical replicates. Differences in expression ratios (ΔCt) among the samples were analysed by ANOVA (LSD test) using StatGraphics (StatPoint Technologies, Inc.). Experiments for qPCR were performed at least three times.

For the microarray experiment, approximately 25 rosettes from three-week old non-inoculated, mock-treated (1 dpi) or *P. cucumerina*-inoculated Col-0 and *aba1-6* plants (1 dpi) were collected (four biological replicates). Plant total RNA was extracted as previously described (Berrocal-Lobo et al., 2002) and further purified using the RNeasy Kit (Qiagen; Germany). RNA quality was tested by using a Bioanalyzer 2100 (Agilent Technologies, US). Three of the four biological replicates were independently hybridized for each transcriptomic comparison. Biotinylated complementary RNA (20 µg) was prepared and the resulting complementary RNA was used to hybridize ATH1 Arabidopsis GeneChips (Affymetrix) using the manufacturer's protocols at the Genomic Unit of the CNB-CSIC (Madrid, Spain). Array images were analysed with GenePix 400B scanner (Molecular Devices) at 10-mm resolution. The images were quantified with GenePixPro 5.1. Gene expression levels were analysed with GeneSpring 7.2 software (Silicon Genetics) and the chip-to-chip signal variation was minimized by normalizing signal intensities to the averaged intensity values of wild type plants using the expression levels of the top 20th percentile of probe sets. Differentially expressed

genes in untreated mutants relative to wild type samples or in *PcBMM*-inoculated relative to the mock-treated samples were identified using two-way analysis of variance and a Benjamini and Hochberg multiple testing correction (Gene Spring 7.2), as described previously (Stein et al., 2006). Genes were considered differentially expressed at $P \leq 0.01$. Up- and down-regulated genes were selected using normalized values (n-fold) higher than 2 or lower than 0.5 relative to control plants.

The Classification SuperViewer Tool (BAR, University of Toronto, Toronto, Canada; http://bbc.botany.utoronto.ca/; Provart et al., 2003) was used to generate an overview of gene ontology classification of the list of differentially regulated genes in *aba1-6* and wild-type plants. Genevestigator Meta-Analyzer tool (www. genevestigator.ethz.ch/; Zimmermann, 2004) was used to analyse expression profiles of differentially regulated genes in the microarray database. Hierarchical clustering of differentially expressed probesets in *aba1-6* and wild-type plants was performed using Pearson Uncentered distance, average linkage and the MeV (Multiexperiment Viewer) application from TM4 software (http://www.tm4.org/; Saeed et al, 2003).

The presence of conserved putative *cis*-regulatory elements in the selected genes was done with Transplanta software (http://bioinfogp.cnb.csic.es/transplanta_dev/), which interrogate a suite of bioinformatic tools (Bioprospector, http://ai.stanford.edu/~xsliu/BioProspector/; DME, http://rulai.cshl.edu/dme/; MEME, http://meme.sdsc.edu/meme/intro.html and Motif Sampler; http://bayesweb.wadsworth.org/gibbs/gibbs.html). Subsequently, TOMTOM (http://meme.sdsc.edu/meme/cgi-bin/tomtom.cgi; Gupta et al 2007) and PLACE databases (Plant Cis-acting Regulatory DNA Elements Database; http://www.dna.affrc.go.jp/PLACE/; Higo et al 1999) were used to identify the statistically overrepresented regulatory elements in the promoters of the genes from each cluster.

Quantification of plant hormones

Three-week old plants were collected before and after treatment (1 dpi) with water or a spore suspension of *PcBMM*. They were frozen immediately in liquid nitrogen, and subsequently freeze-dried. Tissue was then ground to a powder in a tissue lyser (Qiagen, http://www.qiagen.com/). Hormone extractions were performed on 10 mg of freeze-dried tissue as described in Forcat et al. (2008). Data are average of three independent replicates ± SE. Statistical differences among Arabidopsis genotypes were determined by one-way analysis of variance and LSD post hoc test.

Cell wall analysis

For FTIR, precleaned (by solvent extractions) dried leaves from at least 30 three-week-old individual plants per genotype were pooled and homogenized by ball milling. The powder was dried and mixed with potassium bromide and, then, pressed into 13-mm pellets. For each plant genotype, 15 FTIR spectra were collected on a Thermo Nicolet Nexus 470 spectrometer (ThermoElectric Corporation, Chicago) over a range of 4,000 to 400 cm^{-1}. For each spectrum, 32 scans were co-added at a resolution of 4 cm^{-1} for Fourier transform processing and absorbance spectrum calculation, using OMNIC software (Thermo Nicolet, Madison, WI, U.S.A.). Using win-das software (Wiley, New York), spectra were baseline-corrected and were normalized and analyzed, using the PC analysis covariance matrix method (Kemsley et al., 1996). Cell-wall monosaccharides were assayed as alditol acetate derivatives (Stevenson and Furneaux, 1991) by gas chromatography performed on an Agilent 6890N gas chromatograph (Wilmington, DE, U.S.A.), and the results obtained were validated with the method previously described by Gibeaut and Carpita (1991). *Myo*-Inositol (Sigma-Aldrich, St Louis) was added as an internal standard. Cellulose was determined on the fraction resistant to extraction with 2M trifluoracetic acid (TFA) (n = 4) by phenol-sulfuric assay using glucose equivalents as standard (DuBois et al. 1956). Uronic acids were quantified using the soluble 2M TFA fraction (n = 5) (Filisetti-Cozzi and Carpita, 1991). The data were analyzed using two-ways t-Student test p=0.05. All statistical analyses were performed using the statistical software package SPSS 13.0 (SPSS Inc., Chicago).

Accession Number

Expression data from this article was deposited according to MIAME (“Minimum Information About a Microarray Experiment”) standards at ArrayExpress (EMBL-EBI) with the accession number E-MEXP-3733.

Acknowledgements

We thank Dr. Roberto Solano and the Unidad de Genómica from the Centro Nacional de Biotecnología (CNB-CSIC, Madrid, Spain) for technical assistance with transcriptomic analyses, and Dr. Marta de Torres-Zabala (College of Life and Environmental Sciences, University of Exeter, UK) for technical assistance with hormone quantification and critical reading of the manuscript.

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Legends to Figures

Figure 1. Resistance of ABA-deficient and signaling mutants to the necrotrophic fungus *PcBMM.*

Wild-type plants (Col-0, WT) and the *aba1-6*, quadruple *pyr1pyl1pyl2pyl4 (quadr pyr/py)* and triple *abi1-2abi2-2hab1-1* mutants were inoculated with a spore suspension ($4x10^6$ spores/ml) of *PcBMM*. **(A)** qPCR quantification of fungal biomass at 3 days post inoculation (dpi). Specific primers of *P. cucumerina β-TUBULIN* and Arabidopsis *UBIQUITIN21* were used. Values (± standard error, SE) were normalized to *Arabidopsis UBIQUITIN21* and are represented as the average of the n-fold-increased expression compared with the corresponding wild-type plants. Data represent average value of two replicates from one out of three independent experiments performed, which gave similar results. The letters indicate different statistically significant groups (ANOVA, $p \leq 0.05$, Bonferroni Test). **(B)** Macroscopic disease symptoms of inoculated plants at 7 dpi. Fungal hyphae **(C)** and **p**lant cell death **(D)** were visualised in the inoculated leaves by Lactophenol Trypan Blue (TB) 1 dpi. **(E)** Hydrogen peroxide accumulation in inoculated leaves was determined by 3,3'-Diaminobenzidine (DAB) staining at 1 dpi. **(F)** Callose deposition in inoculated leaves (1 dpi) was visualized using aniline blue, and the number of pixels of these depositions were quantified. At least four leaves from each genotype were used for pixel quantification in the two experiment performed that gave similar results **(G)**. Bar represents 10 μm.

Figure 2. Transcriptomic profiles of untreated *aba1-6* mutant and of *PcBMM*-inoculated wild-type plants strongly overlapped.

(A) Hierarchical clustering of the 213 differentially expressed probesets in untreated *aba1-6* mutant compared with wild-type (WT) plants (*aba1*_WT t0). Genes (rows) and experiments (columns) were clustered with the MeV (TIGR) using Pearson Uncentered distance and average linkage. The columns corresponded to untreated *aba1-6* compared to wild-type (WT) plants (*aba1*_wt t0*)*, and *PcBMM*-inoculated wild-type and *aba1-6* plants at 1 dpi compared to their respective mock samples (WT *Pc*_M and *aba1 Pc*_M, respectively). **(B)** Venn diagram showing overlapping of the differentially regulated probesets ($P \leq 0.01$; ANOVA, Benjamini and Hochberg multiple testing correction) in untreated *aba1-6* mutant line (red circle), in *PcBMM*-inoculated wild-type plants (green circle) and in *PcBMM*-inoculated *aba1-6* plants (blue circle). The numbers of differentially regulated probesets in each treatment and of common genes among these treatments are indicated. **(C)** The 128 probesets that were common to

untreated *aba1*-6 plants and *PcBMM*-inoculated wild-type plants were classified using tools of the Bio-Array Resources (BAR) for Plant Biology (http://bar.utoronto.ca/). The normalized score value for each functional class is represented. Normalized score value > 1 indicates over-represented classes in comparison with the whole genome. (**D).** Validation of differentially expressed genes in *aba1*-6 mutants. The expression of *PDF1.2, PR-1, PR-4* and *ORA59* genes was determined by qPCR in untreated wild-type plants (Col-0), *aba1-6* and quadruple *pyr1pyl1pyl2pyl4 (quadr pyr/py)* mutants. Values were normalized to *Arabidopsis UBIQUITIN21* expression levels and for a better comparison with transcriptomic data, were represented as relative n-fold expression to the one in wild-type plants. Bars represent the average (± SD) of two technical replicates. Asterisks indicate differences statistically significant with those of Col-0 plants ($p \leq 0.05$, ANOVA, LSD test). Data are from one out of three independent experiments, which gave similar results.

Figure 3. Hierarchical clustering of genes differentially regulated in untreated *aba1-6* and in *PcBMM*-inoculated wild-type plants.

Hierarchical clustering of the 128 probesets differentially expressed in both *aba1-6* untreated plants (*aba1*_WT t0*)* and in *PcBMM* -inoculated wild-type plants (WT *Pc*_M) with those from treatments of Arabidopsis with different hormones (SA, ACC and MJ; http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp). All data sets were normalized with the RMA method, using Genespring software. Genes (rows) and samples (columns) were clustered with the multi-experiment viewer Tool (MeV; TIGR) using Pearson Uncentered distance and average linkage (Threshold = 0.7). The resulting clusters (I-IV), which are indicated by vertical bars, were further analysed and the results are showed in Supplemental Table 5.

Figure 4. SA, ET and JA are essential for *aba1*-mediated resistance to *PcBMM*

Wild-type plants (Col-0), *aba1-6*, *sid2-1*, *ein2-1*, and *aos* single mutants and *sid2-1aba1*, *ein2-1aba1-6* and *aosaba1-6* double mutants were inoculated with a spore suspension ($4x10^6$ spores/ml) of *PcBMM*. **(A)** Fungal biomass in the inoculated plants (3 dpi) was determined by qPCR using specific primers for *P. cucumerina β-Tubulin*. Values were normalized to *Arabidopsis UBIQUITIN21* expression levels and represented as n-fold increased expression compared to wild-type plants. Data represent average values of two technical replicates (± SE), from one of the three experiments performed that gave similar results. Asterisks (*) indicate the genotypes which show a significant difference in fungal biomass compared with that in wild-type;

(+) indicates the double mutants which show a significant difference in fungal biomass compared with that in *aba1-6* plants; and (Δ) indicate statistical differences in fungal biomass between the double mutants and the corresponding parental line ($p \leq 0.05$, ANOVA, Bonferroni test). **(B)** Macroscopic disease symptoms of *PcBMM* inoculated plants at 7 dpi. **(C)** Expression of *PR-1 (AT2G14610)*, *PR-4 (AT3G04720)*, *LOX2 (AT3G45140)* and *PDF1.2 (AT5G44420),* marker genes of the SA, ET, JA, and ET+JA pathways, respectively, was determined by qPCR on untreated three week-old plants. Values are represented as n-fold increased expression compared to the wild-type. Data correspond to the average (± SD) of two replicates from one representative experiment out of the three performed. Asterisks (*) indicate the single mutants showing a significant differential expression of the gene compared to wild-type, whereas (+) indicates the double mutants showing a significant differential expression of the gene compared to *aba1-6* plants ($p \leq 0.05$, ANOVA, LSD test).

Figure 5. SA, ET and JA pathways do not affect the transpiration rates of *aba1-6* plants.

Sensitivity of wild-type plants (Col-0), *aba1-6*, *sid2-1*, *ein2-1*, and *aos* single mutants and *sid2-1aba1*, *ein2-1aba1-6* and *aosaba1-6* double mutants to water desiccation. Transpiration rates were determined as percentage of loss weight at different time points (from 0 to 180 minutes). Each data point represents the mean of three replicates (± SE). This experiment was repeated three times and gave similar results.

Figure 6. FTIR and biochemical analyses of the cell walls from *aba1-6* mutant and wild-type plants.

(A) FTIR average spectra (n = 15) from three-week-old rosettes of Col-0 wild-type plants (WT) and *aba1-6* mutant. (**B)** Biplot analysis showing a clear separation of *aba1-6* and WT plants. (**C)** Mid-infrared spectra were analysed by the covariance-matrix approach for principal component analysis 1 (PC1). Quantification of cellulose, total uronic acid content (g per mg of dry weight) (**D)** and individual neutral sugars (% Mol) from the non-cellulosic carbohydrate fraction **(E)** from the cell walls of WT plants (black bar) and *aba1-6* (grey bar) mutant. Data represent average values (± SE) of three replicates. Asterisk (*) indicate values statistically different from those of WT plants (t-test); and (ND) not significant differences ($p \leq 0.05$)

Figure 7. The complex regulatory function of ABA in Arabidopsis resistance to the necrotrophic fungus *PcBMM*.

Upon Arabidopsis infection with the fungus the activation of ET, SA, JA pathways and the biosynthesis of Trp-derived metabolites occurs positively regulating (arrows) basal resistance response. ABA signaling negatively regulates (lines) these pathways, and therefore inactivation of ABA-signaling (e.g. *aba1-6* or *quadr pyr/py)* leads to their constitutive activation and an enhanced resistance to the fungus. Previously described interactions among the ET, SA and JA signaling are indicated dotted lines (see text for additional details). The putative function of ABA in regulating wall architecture is indicated by a dotted arrow. Regulation of abiotic stresses responses by ABA seems to be independent on SA, ET and JA.

Table 1. Defence-related genes constitutively up-regulated in the *aba1-6* mutant and induced in wild-type plants after *PcBMM* challenge.

Gene description	Locus	n-fold *aba1*/WT (t=0) [a]	n-fold WT (*Pc*/M) [b]	References
PR-4/HEL (Hevein-Like)	AT3G04720	3.43	3.16	Hejgaard et al., 1992; Potter et al., 1993
PDR8 (Pleiotropic Drug Resistance8)/PEN3	AT1G59870	1.83	1.07	Stein et al., 2006
PR-1 (Pathogenesis Related 1)	AT2G14610	4.10	3.15	Van Loon and Van Strien, 1999
PDF1.2B (Plant Defensin 1.2B)	AT2G26020	5.81	7.78	Thomma et al., 2002
PLP2 (Patatin-Like Protein)	AT2G26560	4.12	3.33	Camera et al., 2005
WRKY33	AT2G38470	3.74	1.15	Zheng et al., 2006
ATTI1 (Trypsin Inhibitor protein 1)	AT2G43510	2.44	1.36	Silverstein et al., 2005
CHI-B; PR-3 (basic chitinase)	AT3G12500	4.13	3.39	Verburg and Huynh, 1991
PRX33 (Peroxidase 33)	AT3G49110	2.02	1.20	Bindschedler et al., 2006; O´Brien et al., 2012
OSM34 (Osmotin 34)	AT4G11650	3.74	2.41	Monteiro et al., 2003
Blue-Copper-Binding protein	AT5G20230	4.03	3.05	Mishina and Zeier, 2007
PDF1.2A (Plant Defensin 1.2A)	AT5G44420	6.20	8.90	Manners et al., 1998; Thomma et al., 2002
RBOHD (Respiratory Burst Oxidase protein D)	AT5G47910	2.26	1.56	Torres et al., 2005
PAD3 (Phytoalexin Deficient 3; CYP71B15)	AT3G26830	6.39	1.16	Glazebrook and Ausubel, 1994; Zhou et al., 1999
CYP81F2 (cytochrome P450)	AT5G57220	4.36	1.50	Bednarek et al., 2009; Clay et al., 2009
ORA59 (Octadecanoid Responsive Arabidopsis AP2/ERF 59)	AT1G06160	3.20	2.88	Pre et al., 2008
PNP-A (Plant Natriuretic Peptide A)	AT2G18660	1,130	5,047	Meier et al 2008
ATGRXS13 (glutaredoxin 13)	AT1G03850	1,553	3.245	Sylvain La Camera et al., 2011
WRKY26	AT5G07100	1.20	1.88	Eulgem and Somssich, 2007

[a] Normalized, average fold ratios *aba1*-6 versus wild-type plant at t=0; [b] Normalized, average fold ratios of *P. cucumerina BMM*-inoculated versus mock-treated wild-type plants; Genes were selected using values of $P \leq 0.01$ (see Methods).

Supplemental Figures

Supplemental Figure 1. Infection of *aba1-6* and wild-type plants with the fungal transformant *PcBMM-GFP*.

Confocal microscopy overlay projections of *PcBMM-GFP* hyphae on leaves of wild-type plants (Col-0) and *aba1-6* mutant at 24 hpi. Fluorescence (green) emitted by *PcBMM-GFP* and by chlorophylls (red) is showed. Scale bar 20 μm.

Supplemental Figure 2. Callose deposition is not essential for Arabidopsis resistance *P.cucumerina BMM*.

Wild-type plants (Col-0, WT), *pmr4 and pmr4 sid2-1* mutants and the hypersusceptible *sid2-1* mutant (Llorente et al., 2005) were inoculated with a spore suspension ($4x10^6$ spores/ml) of *PcBMM*. Disease rating (from 0, not infection; to 5, dead plants) was determined at 12 dpi. Letters indicate different statistical groups, according to ANOVA ($p \leq 0.05$, Bonferroni test).

Supplemental Figure 3. Susceptibility of *wkry33-1* mutant to *PcBMM*.

Wild-type plants (Col-0, WT), *wrky33-1* and the hypersusceptible *agb1-1* mutant (Llorente et al., 2005) were inoculated with a spore suspension ($4x10^6$ spores/ml) of *PcBMM*. Fungal biomass in inoculated plants was determined by qPCR at 3 dpi, using specific primers of *PcBMM β-Tubulin*. Values were normalized to *Arabidopsis UBIQUITIN21* expression levels and represented as n-fold increased expression compared to wild-type plants. Data represent average values of two replicates (± SE) from one out of three independent experiments performed, which gave similar results. Asterisks (*) indicate different statistical groups, according to ANOVA ($p \leq 0.05$, Bonferroni test).

Supplemental Figure 4. Determination of ABA, JA and SA concentrations in leaves of wild-type plants and ABA-defective mutants.

(A) Concentration of ABA, JA and SA levels in untreated three-week old wild type plants, *aba1-6* and *quadr pyr/pyl* mutants. Data are averages (±SE) of three independent experiments. (**B)** n-fold variation of the concentration of hormones in *PcBMM* inoculated plants compared with water-treated plants (1 dpi). Values are averages (±SE) of three independent experiments. Statistical analysis was performed and genotypes with statistical different hormone levels (A) or n-fold induction (B)

compared with those determined in with wild-type plants are indicated (* $p \leq 0.05$ and + $p \leq 0.01$; ANOVA, LSD test).

Supplemental Figure 5. Developmental-associated phenotypes of three-week old single and double mutants defective in different hormone pathways.

Supplemental Figure 6. Biochemical analyses of the cell walls from *quadr pyr/pyl* and *triple abi2-2* mutants and wild-type plants.

Quantification of cellulose, total uronic acid content, and non-cellulosic total sugars from the cell walls of Col-0 wild-type plants (black bar) and *quadr pyr/pyl* and *triple abi2-2* mutants (grey bars). Data represent average values (± SE) of two replicates and are represented as percentage relative to those of Col-0 plants (100%). Asterisks (*) indicate values statistically different from those of Col-0 plants (t-test); and (ND) not significant differences ($p \leq 0.05$).

Supplemental Tables

Supplemental Table 1. Genes differentially regulated in untreated (t=0) *aba1-6* plants compared to wild type plants.

Supplemental Table 2. Genes differentially regulated in wild-type plants inoculated with *PcBMM* (1 dpi) compared to mock-treated plants.

Supplemental Table 3. Genes differentially regulated in *aba1-6* inoculated with *PcBMM* (1 dpi) compared to mock-treated plants.

Supplemental Table 4. Genes differentially regulated in untreated (t=0) *aba1-6* plants compared to wild type plants and in wild type plants inoculated with *PcBMM* (1 dpi) compared to mock-treated plants

Supplemental Table 5. Genes contained in the “Cell-wall” gene ontology category.

Supplemental Table 6. Genes included in clusters I-IV described in Figure 3.

Supplemental Table 7. Effect of ABA treatment on *aba1-1* resistance to *PcBMM*.

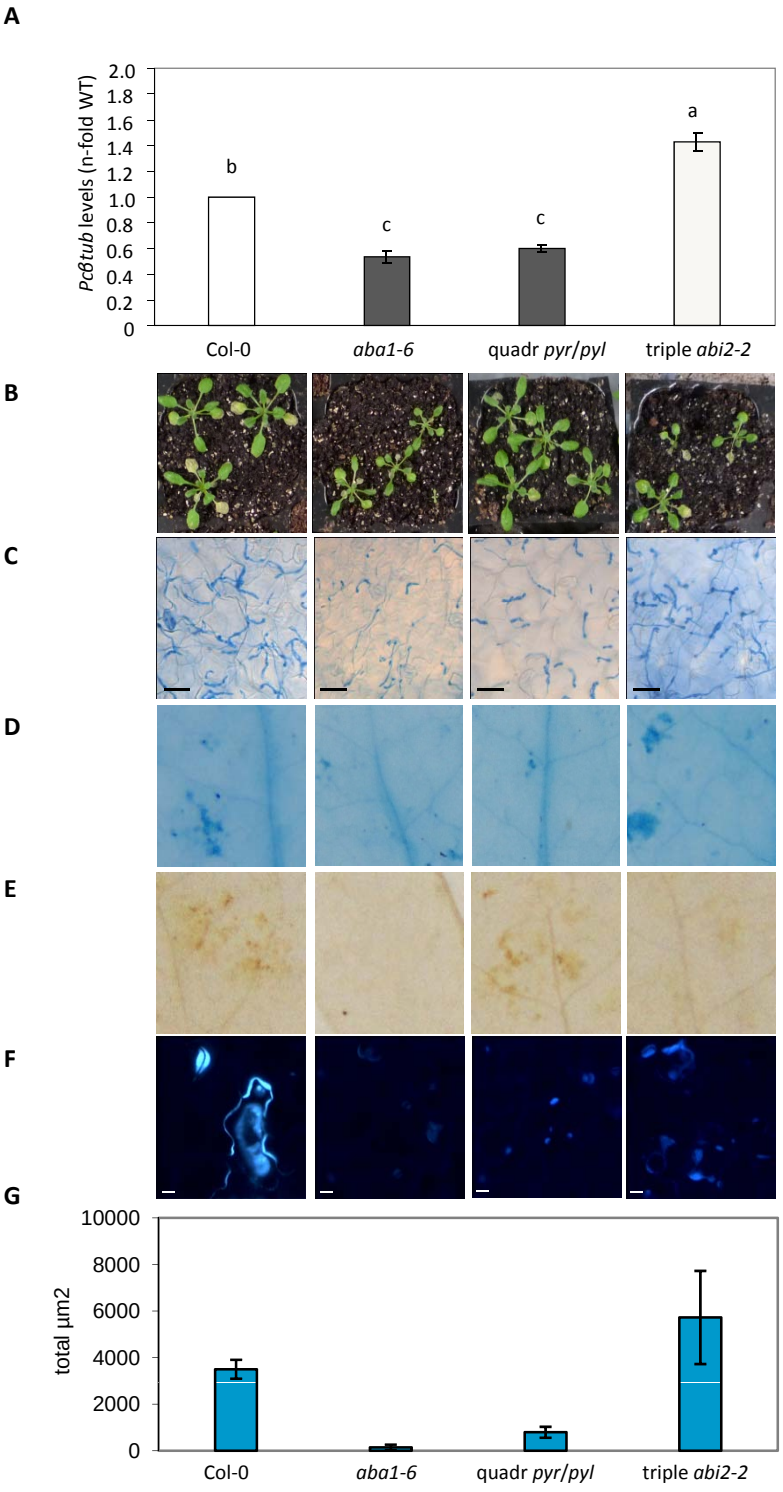


Figure 1. Resistance of ABA-deficient and signalling mutants to the necrotrophic fungus *P. cucumerina BMM.*

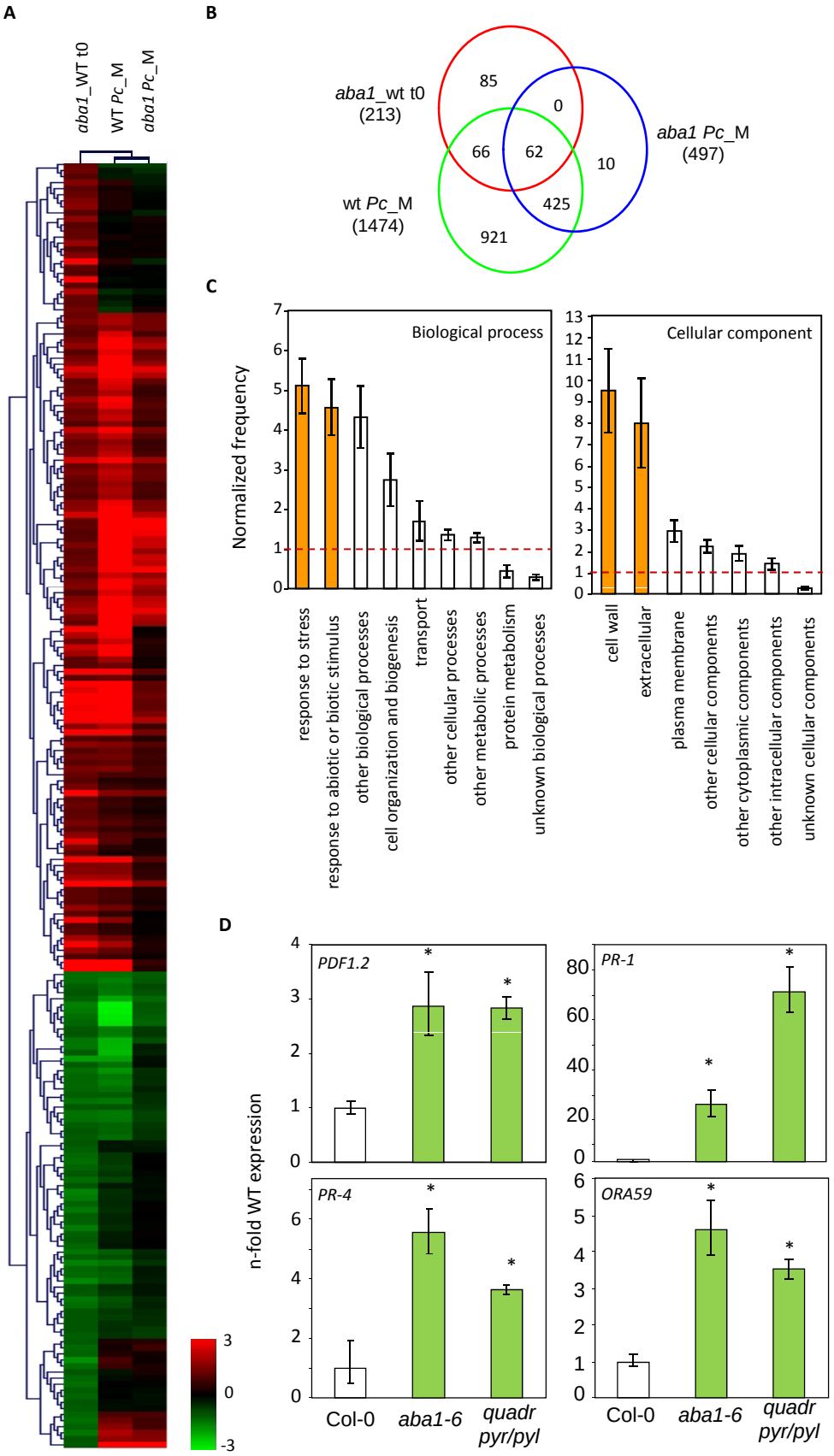


Figure 2. Transcriptomic profiles of untreated *aba1-6* mutant and of *PcBMM* -inoculated wild-type plants strongly overlapped.

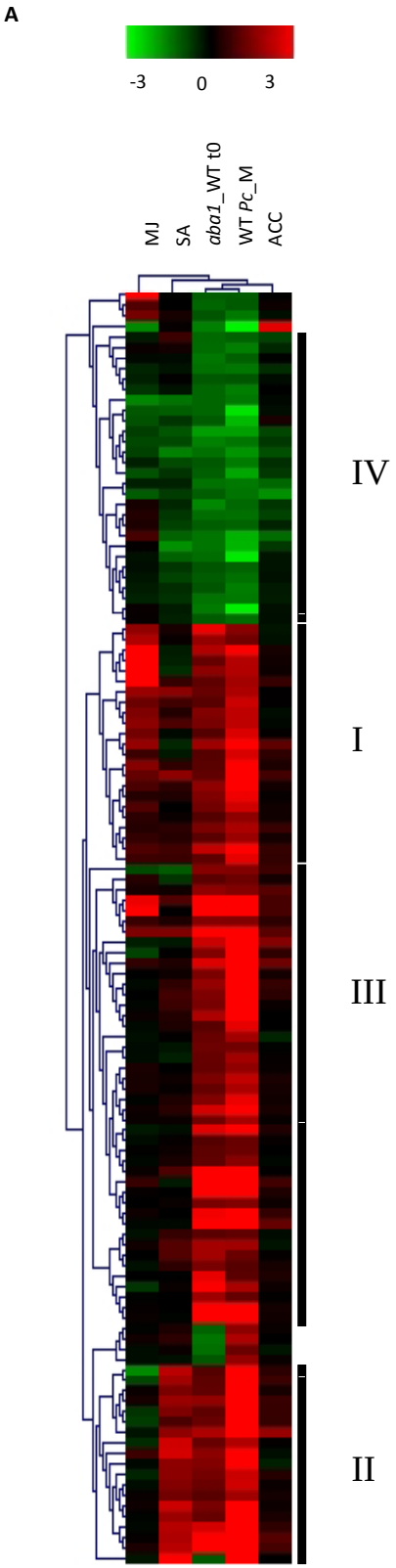


Figure 3. Hierarchical clustering of genes differentially regulated in untreated *aba1-6* and in *PBMM*-inoculated wild-type plants.

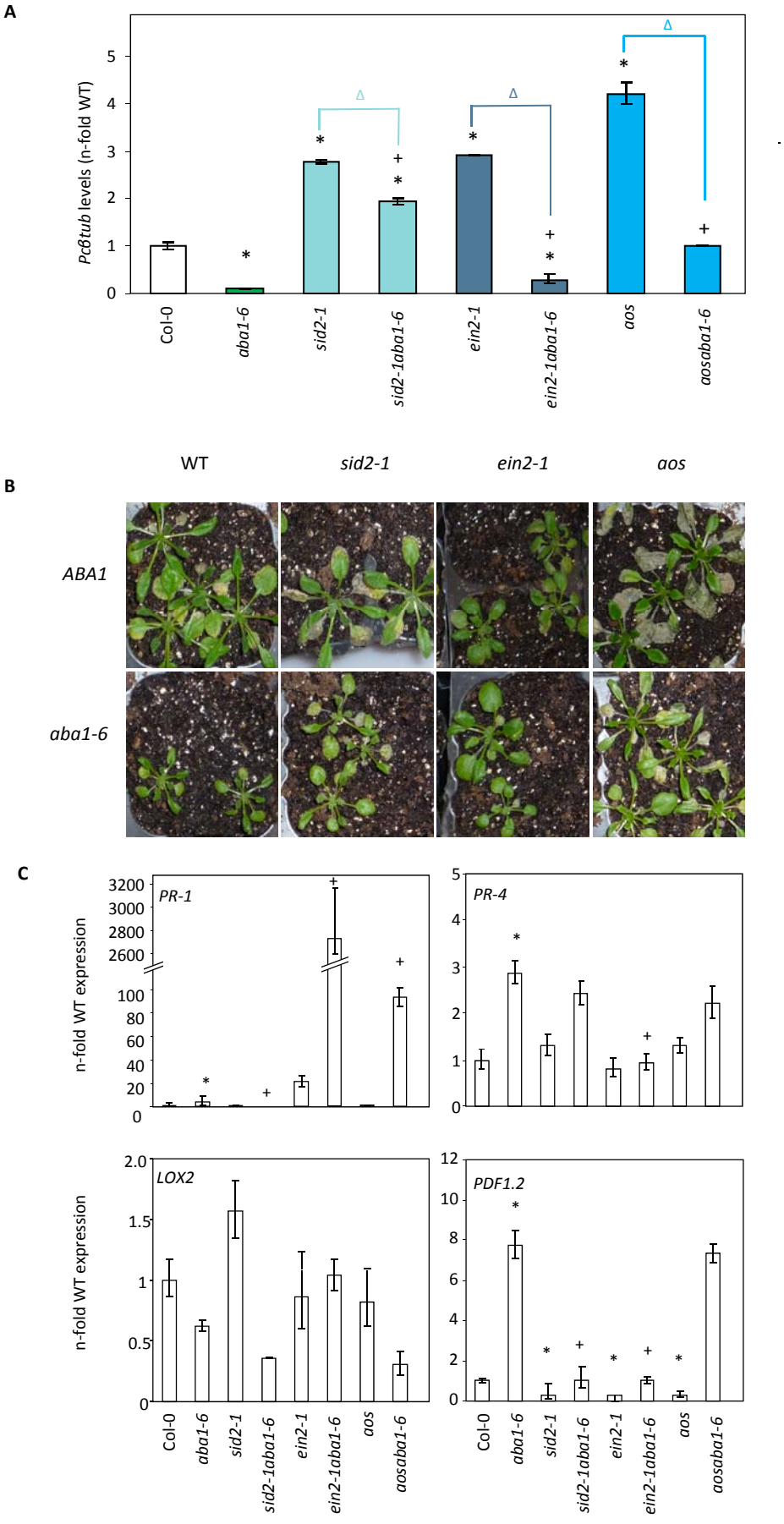


Figure 4. SA, ET and JA are essential for *aba1*-mediated resistance to *PcBMM*

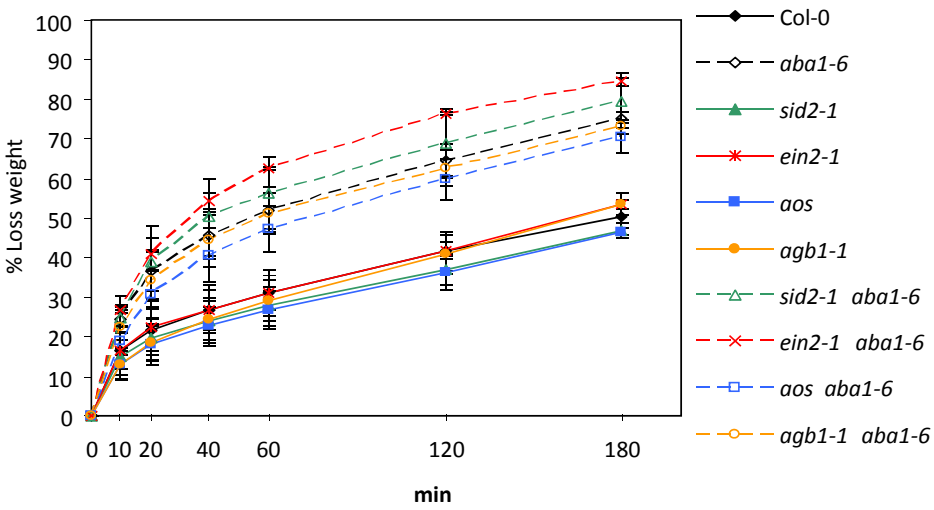


Figure 5. JA, SA and ET do not affect *aba1-6* desiccation phenotype

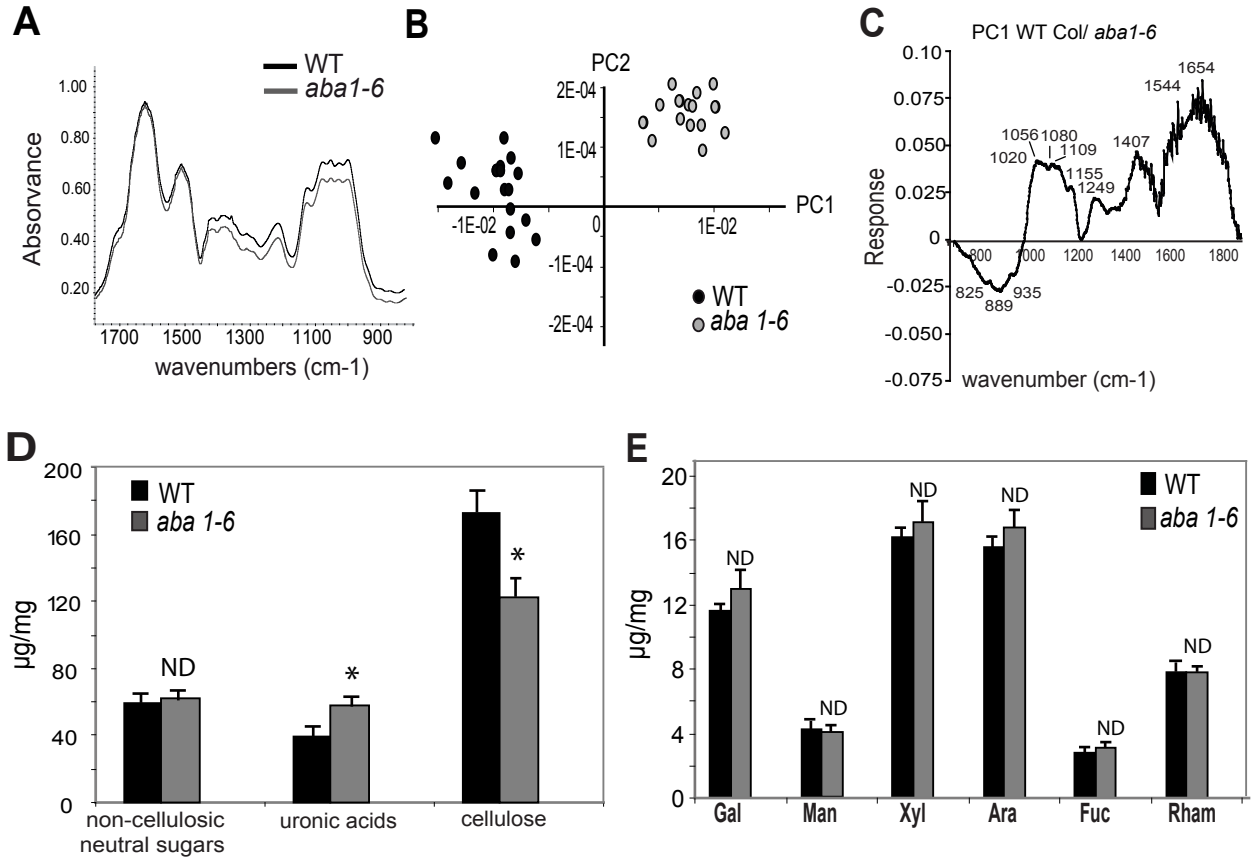


Figure 6. FTIR and biochemical analyses of the cell walls from aba1-6 mutant and wild-type plants.

Figure 7

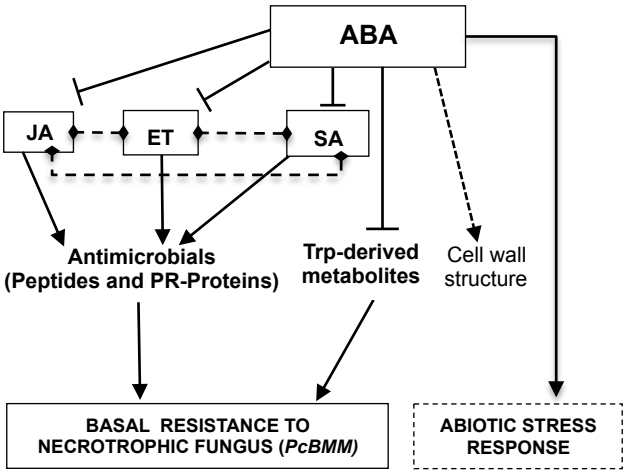
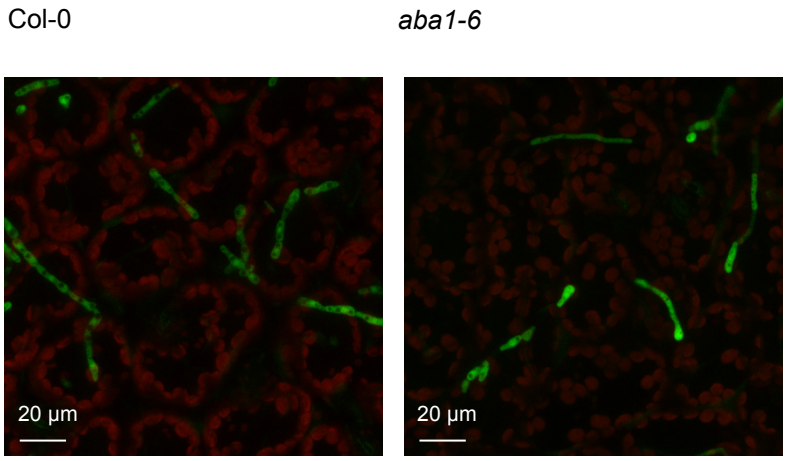
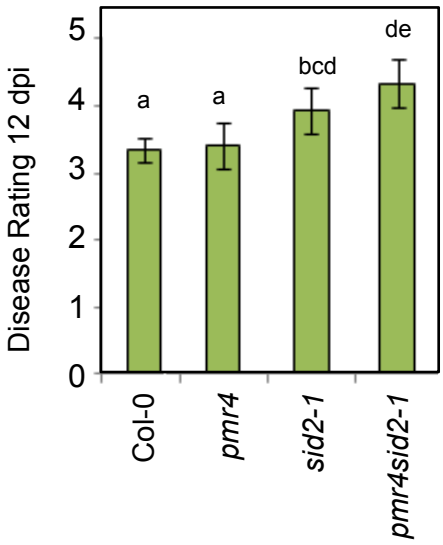


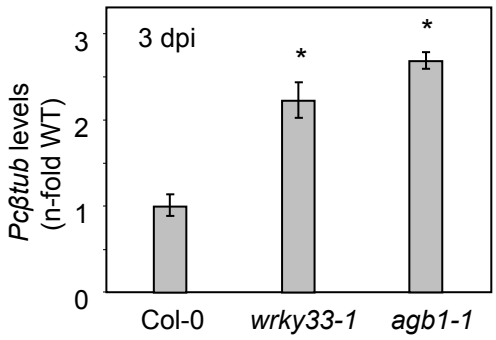
Figure 7. The complex regulatory function of ABA in Arabidopsis resistance to the necrotrophic fungus *PcBMM*



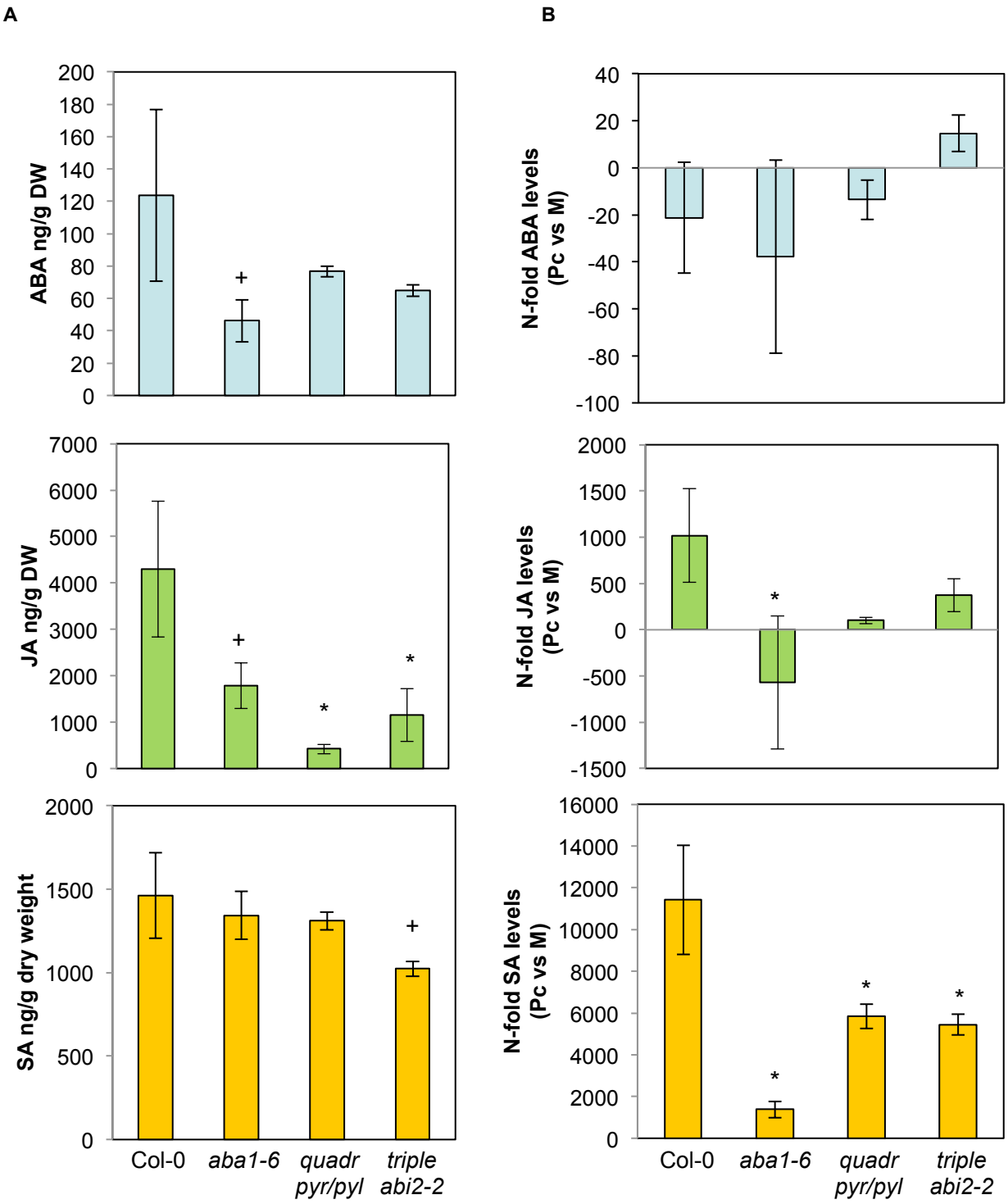
Supplemental Figure 1. *PcBMM-GFP* infection in drop-infected leaves of *aba1-6* plants



Supplemental Figure 2. Callose deposition is not essential for resistance to *P. cucumerina BMM*

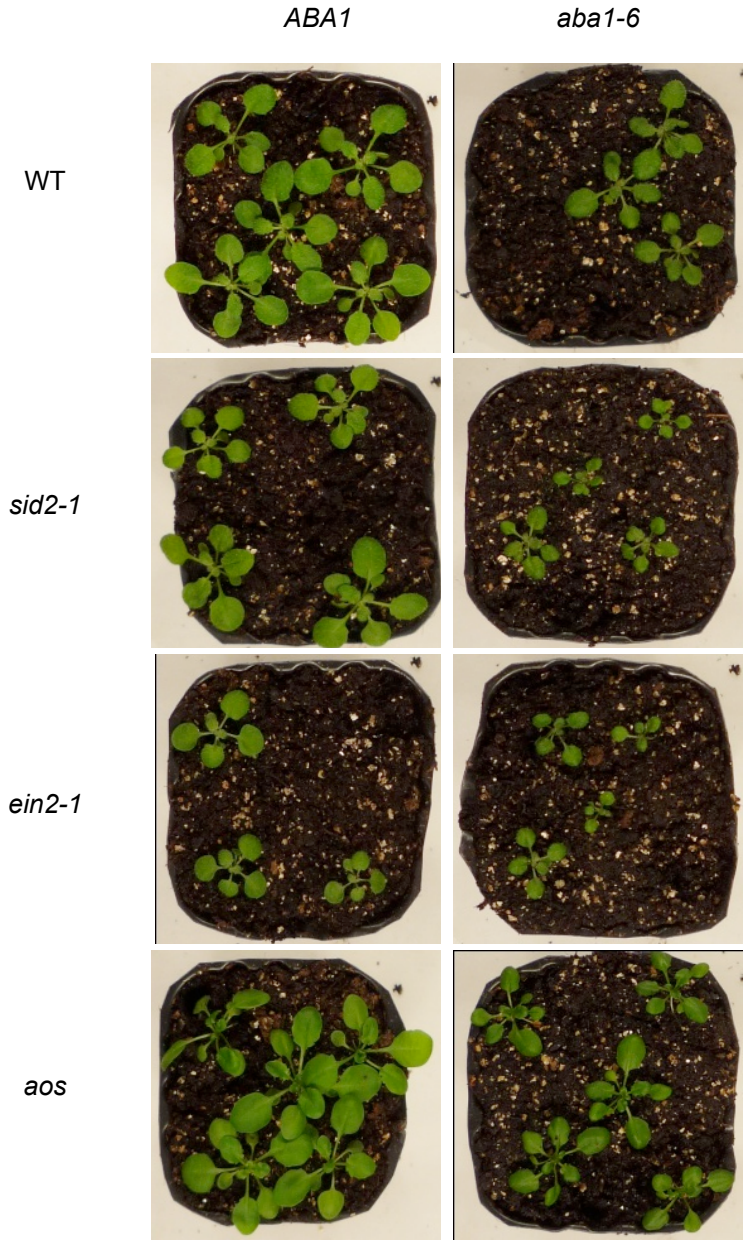


Supplemental Figure 3. Susceptibility of *wkry33-1* mutant to *PcBMM*

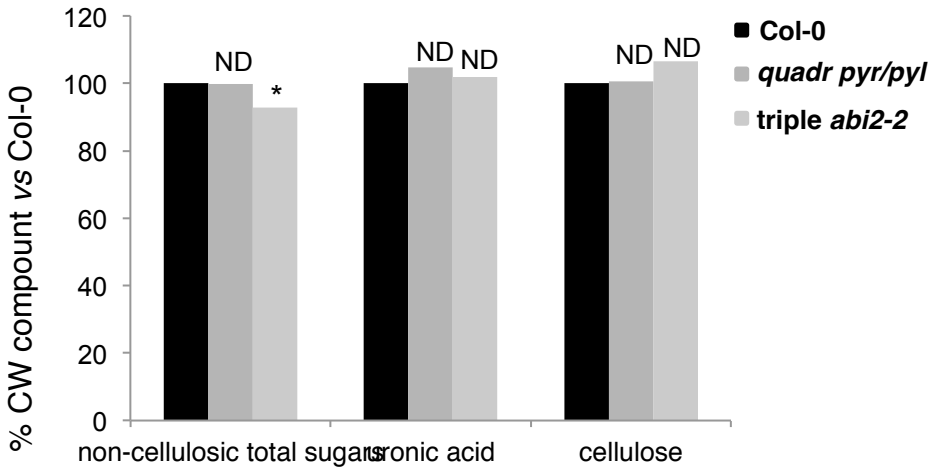


Supplemental Figure 4. Determination of ABA, JA and SA concentrations in leaves of wild-type plants and ABA-defective mutants

Sanchez-Vallet et al.



Supplemental Figure 5. Developmental-associated phenotypes of hormone defective single and double mutants



Supplemental Figure 6. Biochemical analyses of the cell walls from quadr pyr/pyl and triple abi2-2 mutants and wild-type plants.