RESEARCH PAPER

Cell death regulation but not abscisic acid signaling is required for enhanced immunity to Botrytis in Arabidopsis cuticle-permeable mutants

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Abstract
Prevailing evidence indicates that abscisic acid (ABA) negatively influences immunity to the fungal pathogen Botrytis cinerea in most but not all cases. ABA is required for cuticle biosynthesis, and cuticle permeability enhances immunity to Botrytis via unknown mechanisms. This complex web of responses obscures the role of ABA in Botrytis immunity. Here, we addressed the relationships between ABA sensitivity, cuticle permeability, and Botrytis immunity in the Arabidopsis thaliana ABA-hypersensitive mutants protein phosphatase2c quadruple mutant (pp2c-q) and enhanced response to aba1 (era1-2). Neither pp2c-q nor era1-2 exhibited phenotypes predicted by the known roles of ABA; conversely, era1-2 had a permeable cuticle and was Botrytis resistant. We employed RNA-seq analysis in cuticle-permeable mutants of differing ABA sensitivities and identified a core set of constitutively activated genes involved in Botrytis immunity and susceptibility to biotrophs, independent of ABA signaling. Furthermore, botrytis susceptible1 (bos1), a mutant with deregulated cell death and enhanced ABA sensitivity, suppressed the Botrytis immunity of cuticle permeable mutants, and this effect was linearly correlated with the extent of spread of wound-induced cell death in bos1. Overall, our data demonstrate that Botrytis immunity conferred by cuticle permeability can be genetically uncoupled from PP2C-regulated ABA sensitivity, but requires negative regulation of a parallel ABA-dependent cell-death pathway.

Keywords: BOS1, Botrytis cinerea, cell death, cuticle permeable, ERA1, farnesyl transferase, immunity, RNA sequencing.

Introduction
The necrotrophic fungal pathogen Botrytis cinerea is considered to be among the most important plant pathogens, both economically and as a model for research (Dean et al., 2012). Multiple mechanisms determine the outcome of plant–Botrytis interactions, among which stress-hormone-regulated responses have been extensively studied (Mengiste, 2012; AbuQamar et al., 2017). Jasmonic acid (JA) and ethylene signaling are essential and often function together in the activation of immunity to Botrytis (Mengiste, 2012), while salicylic acid (SA) signaling plays a lesser role (AbuQamar et al., 2017).
function of abscisic acid (ABA) signaling in defense against Botrytis is generally considered to be negative (Mengiste, 2012; AbuQamar et al., 2017). Exogenous ABA application enhances Botrytis pathogenicity in a dose-dependent manner (Kettner and Dörfling, 1995; Shaul et al., 1996; Audenaert et al., 2002). Genetic evidence for the role of ABA is primarily based on loss-of-function (ABA-deficient or -insensitive) mutants, which exhibit enhanced immunity to Botrytis (Audenaert et al., 2002; Asselbergh et al., 2007; L’Haridon et al., 2011). However, the evidence is not entirely consistent. We previously tested the Botrytis sensitivity of the ABA hyperaccumulation double mutant cyp707a1 cyp707a3 in Arabidopsis, which is deficient in ABA inactivation, and did not find significantly increased Botrytis susceptibility compared with wild type (Okamoto et al., 2006; Liu et al., 2010; Cui et al., 2016). Furthermore, a recent study reported that exogenous ABA, applied 24 hours prior to infection, could prime the plant defense response and increase Botrytis immunity (Liao et al., 2016). The resolution of this apparent contradiction may lie in the complexity of ABA signaling pathways. ABA signaling branches into many divergent downstream responses, which may differentially participate in regulating immunity to Botrytis.

ABA is required for cuticle formation (Curvers et al., 2010; Cui et al., 2016). Plants with impaired ABA signaling pathways are cuticle permeable. These mutants include the ABA biosynthesis mutants aba deficient2 (aba2) and aba3; the ABA receptor mutants pyrabactin resistance1 (pyr1)/pyr1-like (pyr)/regulatory components of aba receptors (car); and the triple mutant of the three core kinases in ABA signaling, sfr1-related protein kinase (snrk)2.2 snrk2.3 snrk2.6 (here abbreviated to snrk2.236) (Curvers et al., 2010; L’Haridon et al., 2011; Cui et al., 2016). These ABA mutants are also more resistant to Botrytis (L’Haridon et al., 2011; Cui et al., 2016). Although the cuticle acts as a barrier to exclude pathogens (Riederer, 2007), a defective cuticle confers strong immunity to Botrytis (Bessire et al., 2007) that is independent of the canonical antifungal defense signaling pathways, JA, ethylene, SA, and camalexin (Chassot et al., 2007; Serrano et al., 2014). Although the mechanism for this phenomenon remains unknown, it is thought that a permeable cuticle could facilitate early or enhanced perception of Botrytis (Asselbergh et al., 2007; Ziv et al., 2018). Other biological processes may also be involved, including defense activation by cuticle damage-associated molecular patterns, secretion of antifungal compounds, generation of reactive oxygen species (ROS), control of cell death, altered metabolism, and altered foliar microbiome composition (Kliebenstein et al., 2005; Asselbergh et al., 2007; Bessire et al., 2007; Chassot et al., 2007; Curvers et al., 2010; L’Haridon et al., 2011; Seifi et al., 2013; Rinttipatkphong et al., 2016). Thus, this strong enhanced Botrytis immunity may be multilayered, involving several of the processes listed above. Remarkably, no genetic suppressors of this phenotype have been reported.

ABA is also an important regulator of abiotic stresses and cell death. Treating plants with ABA leads to leaf chlorosis and cell death (Fan et al., 1997; Jiang and Zhang, 2001; Takasaki et al., 2015; Zhao et al., 2016). Thus, it is possible that high levels of ABA trigger cell death to promote plant susceptibility to Botrytis. Indeed, mutants with enhanced cell death after ABA treatment were reported to be susceptible to Botrytis.
Fungal cultivation and disease assays

Botrytis cinerea strain BO5.10 was grown on potato dextrose agar. For conidia production, spores with mycelium were collected using forceps into 1/3 strength potato dextrose broth, mixed, filtered, and diluted to 2×10⁶ spores ml⁻¹. For lesion size assays, 3 μl drops were inoculated on to leaves to create Venn diagrams using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny). Putative ERA1 target mutants were typed using PCR genotyping. Mutants were obtained from the European Arabidopsis Stock Centre (http://arabidopsis.info/) or were gifts (see Acknowledgements).

Tissue staining and wounding assays

Dye exclusion experiments were performed with fully expanded leaves from 24-day-old plants, for 20 min with immersion treatment or for 2 h with 5 μl droplets of a 0.05% solution of toluidine blue stain as described by Tanaka et al. (2004). The stained areas were measured with ImageJ according to Cui et al. (2016). Wounding-induced cell death was determined from leaves punctured with a needle. Wounded leaves at 6 days post wounding (dpw) were subjected to trypan blue staining to visualize the cell death. Samples were photographed with a stereomicroscope (Olympus SZX16, Japan) and measured with ImageJ. Each lesion was measured four times through its center. The mean of the four lengths of each wound was taken as the length of spread of cell death. For assessment of H₂O₂ production and Botrytis-induced cell death, spray-infected rosette leaves were stained with 3,3'-diaminobenzidine (DAB; D8001, Sigma–Aldrich) at 16 hours post infection (hpi) and trypan blue (T6146, Sigma) at 36 hpi. The stained leaves were mounted in water to eliminate refraction before being photographed with a stereomicroscope (Olympus SZX16, Japan). The DAB- and trypan blue-stained area and whole leaf area in each sample were measured with ImageJ. The percentage stained area in each sample was measured with ImageJ. The mean of the four lengths of each wound was taken as the length of spread of cell death. For assessment of H₂O₂ production and Botrytis-induced cell death, spray-infected rosette leaves were stained with 3,3'-diaminobenzidine (DAB; D8001, Sigma–Aldrich) at 16 hours post infection (hpi) and trypan blue (T6146, Sigma) at 36 hpi. The stained leaves were mounted in water to eliminate refraction before being photographed with a stereomicroscope (Olympus SZX16, Japan). The DAB- and trypan blue-stained area and whole leaf area in each sample were measured with ImageJ. The percentage stained area in each sample was measured with ImageJ. The mean of the four lengths of each wound was taken as the length of spread of cell death. For assessment of H₂O₂ production and Botrytis-induced cell death, spray-infected rosette leaves were stained with 3,3'-diaminobenzidine (DAB; D8001, Sigma–Aldrich) at 16 hours post infection (hpi) and trypan blue (T6146, Sigma) at 36 hpi. The stained leaves were mounted in water to eliminate refraction before being photographed with a stereomicroscope (Olympus SZX16, Japan). The DAB- and trypan blue-stained area and whole leaf area in each sample were measured with ImageJ. The percentage stained area in each sample was measured with ImageJ.

RNA-seq and data analysis

Botrytis-infected and mock-sprayed plants (five rosettes of each genotype) were collected at 20 and 44 hpi. Three biological replicates were used. All samples were collected at 15.00 h to eliminate the influence of circadian-regulated genes. RNA was extracted with the MiniBEST Universal RNA Extraction Kit (TaKaRa). The total RNA of each sample ranged from 13.8 to 41 μg, with an RNA quality score (RQS) value from 6.7 to 8.5. The RNA-seq was executed by using an Illumina HiSeq 4000 in 150 bp paired-end sequencing. By filtering out adaptors and low-quality reads using Trimmomatic-0.38 (Bolger et al., 2014), we obtained at least 8 Gb clean reads for each sample. The clean data have been uploaded to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/seq/, accession number PRJNA495475). The clean reads were then aligned to the reference A. thaliana genome (release:TAIR10.v32 download from Ensembl Plants) using hisat2 v1.2.0 (Kim et al., 2015) with a modification of intron length for plants (−min-intronlen 20 −max-intronlen 5000). StringTie v1.3.4d (Pertea et al., 2015) was used to construct the new transcripts and generate the merged gene annotations. Finally, to obtain a high confidence of differentially expressed genes (DEGs), at least two of three programs, cuffdiff (Trapnell et al., 2013), edgeR (Robinson et al., 2010), and DESeq2 (Love et al., 2014), were used to determine the DEGs with P≤0.05 and absolute (log₂ fold change) ≥ 1. DEGs were used to create Venn diagrams using Venny 2.1 (https://bioinformatics.cnb.csic.es/tools/venny). Gene Ontology (GO) enrichment was analyzed with the online tools of the Gene Ontology Consortium (http://geneontology.org/page/go-enrichment-analysis) and then the exported data were used to render the GO enrichment pictures in R. For heatmap construction, the heatmap2 package in R was used and then manually adjusted with CorelDRAW (X4) software.

qPCR assay

For confirming gene expression of the RNA-seq data, RNA isolation was performed with the same plant material used for RNA-seq and then treated with DNase I. Real-time quantitative PCR (qPCR) was performed as described by Cui et al. (2016) using YLS8 (AT5G08290), TIP41 (AT4G34270), and PP2A3 (AT1G13320) as reference genes. Primer sequences are given in Supplementary Table S6 at JXB online. For Botrytis growth assays, fungal DNA was extracted from 10 leaf discs (7 mm diameter) from infected plants and qPCR was performed according to Gachon and Sandremann (2004), using specific primers for cutinase A. The raw cycle threshold values were analyzed with Qbase (Hellemans et al., 2007).

Statistical analysis

Statistical analysis of lesion sizes and cell death spread were carried out with scripts in R (version 3.0.3). Using the nlme package, a linear mixed model with fixed effects for genotype, treatment, and their interaction was fitted to the data, plus a random effect for biological repeat. The model contrasts were estimated with the multicomp package, and the estimated P values were subjected to single-step P-value correction. A logarithm of the data was taken before modeling to improve the model fit. The qPCR data were log₁₀-transformed and significance was estimated with a two-tailed Student’s t-test using equal variance. The significance of overlaps between two gene sets was evaluated with Fisher’s exact test in R.

Results

Enhanced immunity to Botrytis in era1-2

Botrytis infections of two ABA-hypersensitive mutants, enhanced response to aba1-2 (era1-2) and pp2c-quadruple (pp2c-q), revealed phenotypes inconsistent with the enhanced susceptibility that would be predicted based on the assumption that ABA acts solely as a negative regulator of Botrytis immunity (Fig. 1A). Disease progression in era1-2 was reduced, measured as smaller lesion size (Fig. 1B) and less Botrytis DNA accumulation (measured with real-time qPCR; Fig. 1C). The extent of cell death was also reduced in era1-2 (Fig. 1D, F). The Botrytis immunity associated with two additional era1 alleles, era1-7 and era1-8, was also enhanced compared with the wild type (Supplementary Fig. S1).

To identify potential mechanisms of enhanced immunity in era1-2, we monitored ROS accumulation during Botrytis infection. Strong H₂O₂ accumulation in Botrytis-infected era1-2 was documented via DAB staining at 16 hpi (Fig. 1E, G). This suggests that the enhanced immunity phenotype of era1-2 may be due to early and/or enhanced ROS production.

ERA1 and cuticle development

An early Botrytis-induced H₂O₂ burst is associated with cuticle permeability (Asselbergh et al., 2007; L’Haridon et al., 2011), prompting us to assay for this phenotype. Toluidine blue staining is a classical cuticle-permeability assay: cuticle-defective leaves fail to exclude the dye, resulting in dark blue staining (O’Brien et al., 1964; Tanaka et al., 2004). The era1-2 mutant stained dark blue, while the wild type and pp2c-q did not (Fig.
To exclude the possibility of second site mutations independent of era1, we confirmed enhanced permeability in mutants in two additional alleles, era1-7 and era1-8 (Goritschnig et al., 2008), using the ABA-insensitive snrk2.236 as a positive control (Fig. 2B, C). The enhanced permeability of the era1-2 mutant was further tested with an immersive staining assay, in which the era1-2 leaf stained mostly dark while the wild-type leaf remained unstained (Fig. 2D). Knockout mutants of ASG2 and CYP85A2, the two known ERA1 substrates that are involved in modulating the ABA response, exhibited normal cuticle permeability (Supplementary Fig. S2), suggesting that the role of ERA1 in cuticle formation is independent of these loci.

Overall, our findings genetically uncoupled ABA sensitivity from Botrytis susceptibility and revealed that ERA1 is required for cuticle formation and negatively regulates Botrytis immunity.

ABA-independent genes deregulated in cuticle-defective mutants

As era1-2 displayed Botrytis immunity, enhanced ABA sensitivity, and a permeable cuticle (Figs 1 and 2), it was utilized as a tool to further explore the relationships between these phenotypes. We used era1-2, snrk2.236, and the cuticle biosynthesis mutant lasc2.3 (Bessire et al., 2007; Tang et al., 2007), to examine global transcriptional changes under Botrytis infection and control conditions. All three mutants are cuticle permeable and Botrytis resistant, whereas their ABA sensitivities are largely different: era1-2 is hypersensitive, lasc2.3 is moderately sensitive, and snrk2.236 is strongly insensitive (Bessire et al., 2007; Fujii and Zhu, 2009; Cui et al., 2016). These three mutants allowed us to identify the effects of ABA sensitivity and reveal the core genes involved in the enhanced Botrytis immunity conditioned by cuticle deficiency.

Mock-treated and Botrytis spore-suspension-infected plants were sampled at 20 hpi. To control for genes under circadian regulation, the second time point was 24 hours later (44 hpi).
Samples of three biological repeats were subjected to RNA-seq analysis. To identify the core genes potentially involved in immunity to *Botrytis* in cuticle-defective mutants (CDMs), we first defined the significantly up-/down-regulated genes in each of the CDMs compared with the wild type. The genes that were mis-regulated in each CDM with fold change ≥2 and \( P \leq 0.05 \) in comparison to the wild type were chosen for further analysis (Supplementary Table S1). The mock-treated CDMs shared a common set of 64 genes with increased expression and seven with decreased expression (Fig. 3A; Supplementary Table S2A, B). Additionally, each mutant had its own unique set of DEGs, but generally *lacs2* and *srk2.236* were more similar to each other than to *era1-2* (Fig. 3A). Under *Botrytis* treatment, the three CDMs had in common 53 genes with increased expression and 25 genes with decreased expression (Fig. 3B; Supplementary Table S2C, D). As in the mock-treated condition, each single mutant had its own unique set of mis-regulated genes (Fig. 3B). Further analysis focused on the genes that were commonly regulated in all three CDMs, as these could be considered as ABA-independent genes and...
may function as CDM-specific components that regulate plant immune responses to Botrytis.

In mock-treated CDMs, multiple receptor-like kinase genes related to defense responses and wounding-responsive genes were up-regulated. (Supplementary Table S2A). In addition, genes required for the pathogenicity of biotrophic or hemibiotrophic pathogens or with negative roles in SA-regulated defense responses were up-regulated (Supplementary Table S2A). For selected genes, RNA-seq data were confirmed with real-time qPCR (Supplementary Fig. S4).

In the Botrytis-treated CDMs, the core up-regulated genes were quite similar to the mock-treated CDMs, as seen in the GO enrichment analysis (Fig. 3C; Supplementary Fig. S3A). The term ‘defense response to bacterium’ was enriched (Fig. 3C). A common set of genes was up-regulated in the mock-treated CDMs and Botrytis-treated CDMs; these included Cysteine-rich receptor-like protein kinase (CRK) family and other kinase genes, salicylic acid signaling genes, and other pathogen-defense-related genes, including the metacaspase gene MC2 (Supplementary Table S2C; Supplementary Fig. S4).

There were also genes specifically up-regulated only in the Botrytis-treated CDMs (Supplementary Table S2C; Supplementary Fig. S4); these included Enhanced Disease Resistance4 and Downy Mildew Resistant6, two SA-regulated genes that are required for plant susceptibility to the biotrophic powdery mildew pathogen (van Damme et al., 2008; Wu et al., 2015). Only a few genes possibly related to Botrytis immunity were up-regulated in the Botrytis-treated CDMs, such as the P450 family member CYP82C2, which is required for the activation of JA signaling and the synthesis of a cyanogenic metabolite to defend against Botrytis (Liu et al., 2010a; Rajnik et al., 2015). In the Botrytis-treated CDMs, 25 genes were identified as the core down-regulated genes (Fig. 3B). Most of these genes are related to functions in the plastid and photosynthesis (Fig. 3D; Supplementary Table S2D).

Overall, the core genes mis-regulated in CDMs were mainly genes previously implicated in pathogen defense responses or SA signaling. This finding indicated that a signal derived from the defective cuticle may lead to the activation of defense responses, ultimately resulting in Botrytis immunity.

**ABA signaling in the transcriptional response to Botrytis**

We further compared the Botrytis-regulated genes at 20 and 44 hpi between the wild type and CDMs. DEGs were identified in each genotype and were compared between genotypes (Fig. 4). At 44 hpi, substantially fewer DEGs were observed in all three CDMs compared with the wild type. This was likely due to the strong immunity in these mutants; that is, there was less dead tissue in the mutants compared with the wild type (Fig. 1; Bessire et al., 2007; Cui et al., 2016).

Col-0 had the most Botrytis-regulated genes, followed by era1-2, lacs2.3, and then snrk2.236 (Fig. 4). The number of Botrytis-regulated genes in the mutants correlated with their ABA sensitivity. This indicated that in the CDMs, the extent of ABA sensitivity still influenced transcriptional responses to Botrytis. To further explore the relation between ABA and Botrytis in transcriptional regulation, we made a comparison between Botrytis-treated CDMs and Col-0 to their corresponding genotype under mock treatment (fold change ≥ 2 and P ≤ 0.05; see also Supplementary Table S3). (A) At 20 h post infection (hpi), there were 11 genes commonly up-regulated (left) and no common genes down-regulated (right) in the Botrytis-treated genotypes. The genes are listed in Supplementary Table S3A. (B) At 44 hpi, there were 61 genes commonly up-regulated (left) and no common genes down-regulated (right) in the Botrytis treated genotypes. The genes are listed in Supplementary Table S3J.

![Fig. 4. Identification of Botrytis-induced differentially expressed genes (DEGs). DEGs were identified in comparisons between Botrytis-treated CDMs and Col-0 to their corresponding genotype under mock treatment (fold change ≥ 2 and P ≤ 0.05; see also Supplementary Table S3). (A) At 20 h post infection (hpi), there were 11 genes commonly up-regulated (left) and no common genes down-regulated (right) in the Botrytis-treated genotypes. The genes are listed in Supplementary Table S3A. (B) At 44 hpi, there were 61 genes commonly up-regulated (left) and no common genes down-regulated (right) in the Botrytis treated genotypes. The genes are listed in Supplementary Table S3J.](https://academic.oup.com/jxb/article-abstract/70/20/5971/5536716)
Expression of previously identified Botrytis-response genes

Extensive research on the interactions between Botrytis and Arabidopsis has identified several positive and negative regulators. We constructed a list of these genes through keyword searches of TAIR (https://www.arabidopsis.org; Supplementary Table S5). The genes related to the JA, ethylene, and PAD3 pathways were excluded, as the Botrytis immunity mediated by cuticle permeability was previously shown to be independent of these signaling pathways (Chassot et al., 2007). Expression values (log2 of the number of transcripts per million) for each gene were used to build a heatmap (Fig. 5). The expression of most genes was similar among genotypes and treatments (Fig. 5), indicating that these genes were not Botrytis inducible or cuticle dependent. However, the expression of some genes, including the autophagy inducer BAG6 (Li et al., 2016), the cell-death regulator BOS1 (Mengiste et al., 2003; Cui et al., 2013), and several pectin methylesterase inhibitors (PMEIs; Lionetti et al., 2017), had increased expression at 44 hpi (Fig. 5). The expression of these genes was genotype dependent; the wild type showed the highest expression, era1-2 showed moderate expression, while snrk2.236 and lacs2.3 showed the lowest expression (Fig. 5). We propose that the expression of these genes may be correlated to cell-death control and lesion development, as Botrytis extracts nutrients from dead and dying tissue. Hence, less expression of these genes would be seen in highly Botrytis-tolerant genotypes. Furthermore, cell-death control is a key factor that determines lesion development in cuticle-defective plants (Asselbergh et al., 2007; Curvers et al., 2010; Seifi et al., 2013). Thus, we chose the bos1 mutant, which exhibited mis-regulated cell-death development (Cui et al., 2013), for further analysis in relation to the other mutants used in this study.

The enhanced Botrytis immunity of era1-2 is BOS1 dependent

We constructed the bos1 era1-2 double mutant to test whether the BOS1-regulated control of cell death was required for the
immunity to *Botrytis* associated with cuticle-defective plants. Interestingly, the enhanced immunity of *era1-2* was fully suppressed in *bos1 era1-2* (Fig. 6A, B). The lesion sizes of *Botrytis*-infected *bos1 era1-2* leaves were markedly lower than those of *era1-2* (Fig. 6A) and were very similar to those of the *bos1* single mutant (Fig. 6B). We previously demonstrated that *bos1* exhibited uncontrolled runaway cell death after wounding (Cui et al., 2013). Thus, we assessed cell death initiated from needle-puncture wounds in *bos1, era1-2*, and the *bos1 era1-2* double mutant. Dead tissue was visualized by trypan blue staining and quantified by measuring the length from the wound edge to the frontier of the spreading dead tissue (Fig. 6C). We found that the spread of cell death in *bos1 era1-2* was slightly but not significantly greater than in *bos1* (Fig. 6C, D). This indicated that the cell-death control conferred by BOS1 is required for the *Botrytis* immunity of the *era1-2* mutant.

Compared with *bos1*, cell death in *bos1 era1-2* was developmentally enhanced in older plants, as *bos1 era1-2* exhibited spontaneous cell death during flowering (Fig. 6E). We observed that once buds started opening, cell death initiated in the buds and then spread along the shoots (Fig. 6E); this led to a sterility phenotype, with no *bos1 era1-2* seeds obtained.

To test whether loss of BOS1 function could suppress the enhanced *Botrytis* immunity of other CDMs, we made the *bos1 lacs2.3* double mutant. The *bos1* mutation restored the *Botrytis* susceptibility of *lacs2.3* to wild-type levels (Fig. 7A, B). Furthermore, runaway cell death in *bos1 lacs2.3* was more extensive than in the wild type, but significantly less extensive than in the *bos1* single mutant (Fig. 7C, D). The early ROS burst present in cuticle-permeable mutants under *Botrytis* treatment was assessed in *bos1 era1-2* and *bos1 lacs2.3*, but was unaltered in both double mutants compared with the respective *era1-2* and *lacs2.3* single mutants (Supplementary Fig. S7). Taken together, these findings indicate that cell-death control is required, but...
the early ROS burst is not sufficient for BOS1-regulated Botrytis resistance.

**ABA sensitivity can be uncoupled from Botrytis immunity**

To explore the relationship between cuticle deficiency, ABA sensitivity, and Botrytis resistance, we analyzed the lesion sizes and cuticle permeability of multiple ABA-related mutants used in this study (Figs. 1, 2, 8) and included normalized data from our previous work (Cui et al., 2016). In these mutants, a linear relationship was observed between cuticle permeability and Botrytis immunity (Fig. 8; $R^2=0.96$, $P<0.01$). However, there was no correlation between ABA sensitivity and immunity. The most ABA-insensitive mutant, 112458, which is impaired in six ABA receptors (Gonzalez-Guzman et al., 2012), was more Botrytis-susceptible than the ABA-hypersensitive mutant era1-2 (Fig. 8). The lacs2.3 mutant, which was moderately impaired in ABA signaling (Wang et al., 2011), was more resistant than the snrk2.236 mutant, which is severely impaired in ABA signaling (Fig. 8). These data genetically demonstrate that Botrytis resistance associated with cuticle permeability could be uncoupled from ABA sensitivity.

In the bos1 background, Botrytis resistance was not dependent on cuticle permeability (Fig. 8; $R^2=0.52$, $P=0.49$). Cuticle permeability in era1-2 and bos1 era1-2 was similar, as was the case for lacs2.3 and bos1 lacs2.3 (Fig. 8), indicating that BOS1 was not involved in cuticle formation. The lesion sizes of bos1, bos1 era1-2, and bos1 lacs2.3 were proportional to the severity of their wound-induced spreading cell death phenotypes (Fig. 6C; Fig. 7C). This suggested that BOS1-regulated cell death was genetically required for cuticle-related Botrytis immunity.

**Role of cell-death control in enhanced Botrytis immunity**

We previously demonstrated that impaired ABA signaling or biosynthesis could largely suppress the runaway cell-death phenotype of bos1 (Cui et al., 2013). To test the correlation between wound-induced cell death and Botrytis lesion size, the aba3 bos1 and abi1-1 bos1 double mutants were further examined with Botrytis droplet infection. Similar to wound-induced cell death (Cui et al., 2013), Botrytis-induced lesions in aba3 bos1 and abi1-1 bos1 were significantly smaller than in bos1 (Fig. 9A). Spray infection with Botrytis caused enhanced necrosis in bos1, which was also significantly reduced in the aba3 bos1 and abi1-1 bos1 double mutants (Fig. 9B). We plotted Botrytis-induced lesion sizes against the extent of wounding-induced cell death (Fig. 9C); this includes meta-analysis of normalized wound-induced cell death data from Cui et al. (2013), and found a significant correlation ($R^2=0.78$, $P<0.05$; Fig. 9C). Thus, the extent of cell death regulated by BOS1 plays a determinant role in the regulation of plant Botrytis sensitivity.

Taken together, our data illustrate that the Botrytis resistance in CDMs likely consists of several layered biological mechanisms. Initial resistance is conferred by pre-activated defense signaling, including increased expression of defense-related genes (Fig. 3C; Supplementary Table S2; Fig. 10). During early infection, enhanced ROS production (Fig. 1E; L’Haridon et al., 2011) may augment plant defenses. At a later stage of infection, control of cell death becomes more important and determines the development of Botrytis-induced necrosis (Fig. 10). On the leaves of Botrytis-infected CDMs, cell death is dramatically attenuated at the frontier of the lesions, resulting in smaller lesion sizes compared with the wild type (Curvers et al., 2010; AbuQamar et al., 2017). This control of Botrytis-induced cell death requires BOS1 (Figs. 5–7, 10).

**Discussion**

Understanding the complexities of ABA signaling at the intersection between cuticle deficiency and Botrytis responses is challenging: ABA-deficient and -insensitive mutants are generally cuticle defective (Cui et al., 2016; Martin et al., 2017), while cuticle biosynthesis mutants, such as bgd/ced1, lcr/cyp86a8, gpat4 gpat8, and lacs2-1, exhibit altered stress-induced accumulation of ABA-biosynthesis transcripts and enhanced sensitivity to osmotic stress (Wang et al., 2011). Here we provide new genetic data that refines the roles of pathways downstream of ABA in plant–Botrytis interactions.

**The role of ABA signaling in plant–Botrytis interactions**

Our comparisons between mutants with different levels of ABA sensitivity, cuticle permeability, and response to Botrytis (Fig. 8) demonstrated that cuticle permeability, but not ABA sensitivity, determines Botrytis resistance in CDMs. However, the role of ABA in wild-type plants is complicated during Botrytis infection. The application of ABA to plants results in enhanced Botrytis susceptibility (Audenaert et al., 2002), while Botrytis also secretes ABA (Siewers et al., 2004, 2006; Amselem et al.,
Fig. 9. Botrytis susceptibility of bos1 double mutants was positively correlated with the extent of wound-induced cell death. (A) Suppressors of spreading cell death in bos1 (aba3-1 and abi1-1) also suppressed the Botrytis susceptibility of bos1. Lesion sizes from four independent experiments were combined and analyzed in a linear mixed model with a single step P-value adjustment. Error bars represent the SE of means (n=48 in total). Different letters above the bars indicate significant differences (P<0.05). (B) Representative symptoms of plants sprayed with Botrytis spore suspensions at 3 days post infection. Scale bar=1 cm. (C) Botrytis sensitivity correlated with the extent of wound-induced cell death in bos1 double mutants (R²=0.75, P=0.017). The blue dotted line shows a linear correlation between the extent of wound-induced spreading cell death and the Botrytis-induced lesion sizes. The genotypes indicated with blue squares were examined in this study. The spread of cell death of the genotypes indicated with grey squares are from previously published data (Cui et al., 2013), which were calculated with normalization to the reference values of the wild type and bos1. All experiments were repeated three to six times.

Exogenous ABA application enhances the development of cell death symptoms (Fan et al., 1997; Cui et al., 2013; Takasaki et al., 2015; Zhao et al., 2016). The GO categories ‘response to oxidative stress’ and ‘response to hydrogen peroxide’ were enriched in the genes regulated by both ABA treatment and Botrytis infection at both the early and later time points (Supplementary Fig. S6). These categories are integrated in the process of cell death (Overmyer et al., 2003; Van Breusegem and Dat, 2006). Thus, one role for ABA in responses to Botrytis could be to alter the extent of cell death. We explored this further utilizing the bos1 mutant, which displays enhanced cell death upon ABA application and increased ROS production after Botrytis infection and wounding (Mengiste et al., 2003; Kraepiel et al., 2011; Cui et al., 2013). The abi1-1 and aba3 mutants suppressed the severe Botrytis susceptibility of bos1. Proportionally, these mutants also suppressed the extent of wound-induced spreading cell death in bos1 (Fig. 9C). Thus, a linear correlation between Botrytis sensitivity and ABA-regulated cell death was demonstrated. This was also supported by the symptoms shown by lacs2.3 bos1 and ena1 bos1. The lacs2 mutant is deficient in one step of cuticle biosynthesis, but also exhibits a secondary phenotype of deficient ABA biosynthesis (Wang et al., 2011). The lacs2.3 mutant attenuated both the runaway cell death and Botrytis susceptibility phenotypes of bos1 (Fig. 7). However, ena1-2 had no effect on either of these phenotypes in ena1-2 bos1 (Fig. 6). This genetic evidence supports the hypothesis that ABA can influence Botrytis sensitivity through the regulation of cell death. Since ABA sensitivity had little effect in the Col-0 background (Fig. 8) but was linearly correlated with Botrytis susceptibility in the bos1 background (Fig. 9C), we propose that ABA signaling could affect plant sensitivity to Botrytis only when it is activated at levels high enough to trigger cell death.

Resistance trade-offs in cuticle-permeable mutants

Cuticle permeability confers effective resistance to certain necrotrophic pathogens, such as Botrytis, but also enhances susceptibility to other pathogens (Laźniewska et al., 2012; Serrano...
Roles of abscisic acid in *Botrytis* susceptibility

![Diagram of defense responses to *Botrytis*](https://example.com/diagram.png)

**Fig. 10.** Model of layered defense responses to *Botrytis* in cuticle-deficient plants. Cuticle permeability itself leads to increased expression of defense genes related to pathogen perception and salicylic acid signaling. In addition, a permeable cuticle enhances the production of reactive oxygen species (ROS), which act as signaling molecules and regulators of cell death (see also L’Haridon et al., 2011). BOS1 is required to maintain cell viability and contain *Botrytis*-induced lesion expansion. The left panel represents the initial resistance and the middle panel represents a later stage of infection. The right panel illustrates signaling relationships under normal conditions, with an intact cuticle and the absence of pathogens, in which ERA1 attenuates ABA signaling and independently promotes cuticle formation via an unknown mechanism.

Other genes displaying higher expression in CDMs act as negative regulators in SA signaling. These genes include **ERA1** (Ziegelhoffer et al., 2000), which was isolated and extensively studied for its functions related to development and abiotic stresses (Galichet and Gruissem, 2003). A few studies have also explored its role in responses to pathogens including the hemibiotrophic bacterial pathogen *P. syringae* pv. *maulia* and the biotrophic oomycete pathogen *H. parasitica* (Goritschnig et al., 2008). ERA1 was suggested to play a role in the interaction between ABA and defense signaling, such as SA (Goritschnig et al., 2008). ERA1 adds a farnesyl to its typical substrate proteins, which contain a CAAX motif at the C-terminal (Galichet and Gruissem, 2003). ERA1 has over 700 potential targets for farnesylation; this presents a considerable challenge to identifying the relevant protein that acts as a regulator of *Botrytis* defense and/or cuticle formation (Goritschnig et al., 2008; Norrington et al., 2016). At the same time, the existence of over 700 potential targets makes the identification of the ERA1 substrate that regulates cuticle formation a considerable challenge (Ziegelhoff et al., 2000). Thus, some caution should be taken in the interpretation of *ena1* phenotypes compared with other permeability mutants. In the future, identification of the ERA1 substrate that regulates cuticle formation, for example, with a protein purification approach (Dutilleul et al., 2016), could lead to the identification of the mechanisms that...
regulate cuticle formation. However, even without knowledge of this ERA1 target, the robust ABA hypersensitivity and cuticle permeability of era1 make it a useful tool to explore the interactions between ABA, cell death, cuticle permeability, and responses to Botrytis.

In this study, we reported the functions of ERA1 in cuticle formation and Botrytis resistance, genetically uncoupled plant ABA sensitivity from Botrytis sensitivity, and identified bos1 as the first suppressor of Botrytis immunity in CDMs. These results support the hypothesis that ABA can promote susceptibility to Botrytis infection via the regulation of plant cell death, and provide a framework for future work on the role of ABA in the regulation of plant–Botrytis interactions.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Increased Botrytis immunity in era1-7 and era1-8.

Fig. S2. Cuticle permeability of known ERA1 substrates.

Fig. S3. DEGs in cuticle-defective mutants compared with Col-0 under mock and Botrytis treatments.

Fig. S4. Real-time quantitative reverse transcription–PCR data.

Fig. S5. Significant overlaps between ABA- and Botrytis-regulated genes.

Fig. S6. GO enrichment analysis of genes regulated by both ABA and Botrytis.

Fig. S7. Early Botrytis-induced ROS in era1-2 and lac2.3 were not attenuated by bos1.

Table S1. DEGs identified in comparisons between genotypes (CDMs versus Col-0) and treatments (Botrytis versus mock).

Table S2. Core genes common to the DEGs of each CDM.

Table S3. Botrytis-responsive genes of each genotype.

Table S4. Common genes regulated by both ABA and Botrytis.

Table S5. Gene information and expression values used for Fig. 5.

Table S6. Primers used in this work.

**Data deposition**

The RNA-seq data presented in this paper have been deposited in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA495475.

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**References**


