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Absence of Cu–Zn superoxide dismutase BCSOD1 reduces *Botrytis cinerea* virulence in Arabidopsis and tomato plants, revealing interplay among reactive oxygen species, callose and signalling pathways

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SUMMARY

Plants activate responses against pathogens, including the oxidative burst. Necrotrophic pathogens can produce reactive oxygen species (ROS) that benefit the colonization process. Previously, we have demonstrated that tomato plants challenged with *Botrytis cinerea* accumulate ROS and callose, together with the induction of genes involved in defence, signalling and oxidative metabolism. Here, we studied the infection phenotype of the Δ*bcsod1* strain in both tomato and Arabidopsis plants. This mutant lacks *bcsod1*, which encodes Cu–Zn superoxide dismutase (SOD). This enzyme catalyses the conversion of superoxide ion (O_2^-) into hydrogen peroxide (H_2O_2). ROS play a protective role and act as signals in plants. Δ*bcsod1* displayed reduced virulence compared with wild-type B05.10 in both species. Plants infected with Δ*bcsod1* accumulated less H_2O_2 and more O_2^- than those infected with B05.10, which is associated with an increase in the defensive polymer callose. This supports a major role of fungal SOD in H_2O_2 production during the plant–pathogen interaction. The early induction of the callose synthase gene *PMR4* suggested that changes in ROS altered plant defensive responses at the transcriptional level. The metabolites and genes involved in signalling and in response to oxidative stress were differentially expressed on Δ*bcsod1* infection, supporting the notion that plants perceive changes in ROS balance and activate defence responses. A higher O_2^-/H_2O_2 ratio seems to be beneficial for plant protection against this necrotroph. Our results highlight the relevance of callose and the oxylipin 12-oxo-phytodienoic acid (OPDA) in the response to changes in the oxidative environment, and clarify the mechanisms that underlie the responses to *Botrytis* in Arabidopsis and tomato plants.

Keywords: *Arabidopsis thaliana*, *Botrytis cinerea*, callose, OPDA, ROS, SOD, *Solanum lycopersicum*.

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INTRODUCTION

In the environment, plants can suffer the invasion of a wide range of pathogens. When an interaction occurs, plants activate their signalling pathways to trigger defence responses to limit pathogen expansion. Plant defence responses include the oxidative burst, an early event considered to be the largest element of resistance to plant diseases (Govrin and Levine, 2000), and which is associated with other mechanisms of resistance. Reactive oxygen species (ROS) are released and lead to the accumulation of harmful radicals, such as singlet oxygen, hydroxyl (OH^-) and other oxidizing compounds, e.g. hydrogen peroxide (H_2O_2), which help to limit pathogen advance (de Gara *et al.*, 2003). These molecules can also act as stress signals and trigger programmed cell death (Asselbergh *et al.*, 2008). Phytohormones also play a key role in plant responses to pathogen attack by the signalling and coordination of pathways, but these pathways form part of a complex network that is difficult to interpret. Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play an important role in defence responses against phytopathogens. The SA signalling pathway is mainly activated against biotrophic pathogens, whereas JA and ET signalling pathways are associated with resistance against necrotrophic pathogens (Beckers and Spoel, 2006; Zimmerli *et al.*, 2004). However, SA can antagonize JA signalling, and vice versa (Glazebrook, 2005), and the balance between these and other hormones finally determines the outcome of the infection (Caarls *et al.*, 2015; Grant *et al.*, 2009). The pathogen can also manipulate hormone signalling, but the host can attenuate this manipulation. Hence, the fine crosstalk between these pathways generates variable responses according to the challenging pathogen (Glazebrook, 2005; Pieterse *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011). On infection, the penetration site of the pathogen reinforces papilla formation, whose main component is callose (Smart *et al.*, 1986). Callose is a polymer of glucose residues joined by 1,3-β-D links, which is deposited between the plasma membrane and the inner face of the primary cell wall. Its accumulation occurs against the attack

of some pathogens and forms part of the hypersensitive response (HR) (Donofrio and Delaney, 2001; Ryals *et al.*, 1996). Papilla formation is an early response that is induced before later responses which may require the transcriptional activation of genes involved in complex signalling pathways (Voigt, 2014). Although callose is not effective against all pathogens, its deposition is a marker of the activation of defence responses (Hauck *et al.*, 2003).

The necrotrophic fungus *Botrytis cinerea*, responsible for grey mould, causes cell death of its host to aid its colonization. Unlike other plant-pathogenic fungi, *Botrytis* is able to enter directly into healthy tissue and produce areas of necrotic lesions, which spread rapidly throughout the tissue if plant defences are weak (Williamson *et al.*, 2007). Callose accumulation can hinder the expansion of *B. cinerea* in tomato and Arabidopsis (Finiti *et al.*, 2013; Flors *et al.*, 2007), but the pathogen can exploit SA and JA antagonism as a strategy to cause disease development (El Oirdi *et al.*, 2011). *Botrytis* also contributes to the oxidative burst on infection and requires plant responses to achieve complete pathogenicity (Heller and Tudzynski, 2011). This has been tested in *Arabidopsis* plants that lacked HR, where the virulence of *Botrytis* was diminished (Govrin and Levine, 2000). However, the contribution of both plants and pathogens to ROS production remains unclear. ROS are harmful for the pathogen, but also play a role in the differentiation process in this pathogenic fungus (Viefhues *et al.*, 2014). *Botrytis*, like other pathogens, possesses multiple enzymes that are involved in ROS-generating systems. One of these is superoxide dismutase (SOD), which belongs to a family of enzymes that neutralize ROS effects by catalysing the conversion of O_2^- into H_2O_2. Δ*bcsod1* is deficient in a gene that encodes the major Cu–Zn SOD of *B. cinerea* (Rolke *et al.*, 2004). The radial growth and variability in the size of necrotic lesions in French bean leaves are reduced in this strain compared with wild-type (WT) strains (Patel *et al.*, 2008; Rolke *et al.*, 2004).

In this work, we characterized the infection phenotype of the Δ*bcsod1* mutant in *Arabidopsis* and tomato plants (*Solanum lycopersicum* cv. Ailsa Craig) compared with the WT strain B05.10. Our results show that the absence of *bcsod1* reduces the infectivity of the necrotroph compared with the WT strain in both cultures. On infection, the accumulation of superoxide ion (O_2^-) increases and that of H_2O_2 decreases. The imbalance in ROS is associated with increased pathogen-induced callose and with changes in the hormone profile and gene expression. The increase in both 12-oxo-phytodienoic acid (OPDA) accumulation and regulated genes is particularly interesting, confirming the role of this oxylipin in plant responses to pathogens. The differential expression of the genes involved in signalling, defence and the response to oxidative stress demonstrates that plants perceive changes in the ROS environment by the activation of responses that reduce plant susceptibility.

RESULTS

Δ*bcsod1* mutant shows significantly reduced virulence in Arabidopsis and tomato plants

Arabidopsis thaliana plants were inoculated with 10^5 spores/mL of the B05.10 (WT) or Δ*bcsod1* strain, and the necrotic lesion areas were determined at 48, 72 and 96 h post-inoculation (hpi). The Δ*bcsod1* mutant showed reduced virulence compared with WT (see Fig. 1A,B). Tomato plants are more susceptible than Arabidopsis plants to B05.10 and were challenged with 5×10^4 spores/mL of the B05.10 (WT) or Δ*bcsod1* strains. Infection tests with the Δ*bcsod1* mutant in young tomato plants showed the same phenotype, with significantly reduced lesions at 72 hpi (Fig. 1C,D).

Lack of *bcsod1* alters ROS accumulation on infection

SOD is an H_2O_2-generating enzyme that catalyses the conversion of O_2^- into H_2O_2. We determined the effect of Δ*bcsod1* infection on ROS in Arabidopsis and tomato plants by analysis of the accumulation of H_2O_2 and O_2^- by 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively. Arabidopsis plants challenged with B05.10 (WT) exhibited brown spots, which are indicative of H_2O_2 accumulation, around the infection site and mainly in the central area where most of the mycelium is concentrated. Plants infected with Δ*bcsod1* presented reduced H_2O_2 at both locations at 24, 48 and 72 hpi compared with WT (Fig. 2A). Quantitative analysis showed that H_2O_2 accumulation in plants infected with Δ*bcsod1* was almost two-fold lower than in those infected with B05.10 (Fig. 2B). O_2^- analysis revealed that the corresponding blue spots accumulated mostly in the central area of the infection site in plants challenged with B05.10. The plants infected with Δ*bcsod1* showed increased superoxide accumulation at this location at 24, 48 and 72 hpi (Fig. 2C), which was around two-fold greater than in WT (Fig. 2D). Tomato plants inoculated with Δ*bcsod1* exhibited a similar reduction in H_2O_2 at the infection site to that observed in Arabidopsis plants, which was associated with an increase in O_2^- (Fig. 2E,F). These results demonstrate that the absence of fungal SOD alters the oxidative environment in Arabidopsis and tomato plants in association with reduced virulence of Δ*bcsod1*. As *B. cinerea* and the host plant actively produce ROS during infection, we examined whether internal oxidative stress in Δ*bcsod1* before inoculation would reduce fungal virulence and cause the ROS imbalance observed in the infected plants. Quantification of H_2O_2 and O_2^- by DAB and NBT tests *in vitro* showed reduced H_2O_2 and increased O_2^- in Δ*bcsod1* relative to WT (Fig. 3A,B). The addition of diphenyleneiodonium (DPI), an inhibitor of flavocytochromes, such as NADPH oxidases, prevented NBT staining, suggesting that superoxide ions are generated enzymatically by flavoenzymes (Fig. 3B). To examine whether inappropriate ROS production of the Δ*bcsod1* mutant is responsible for its inability to infect, an antioxidant was added in a pathogenicity test.

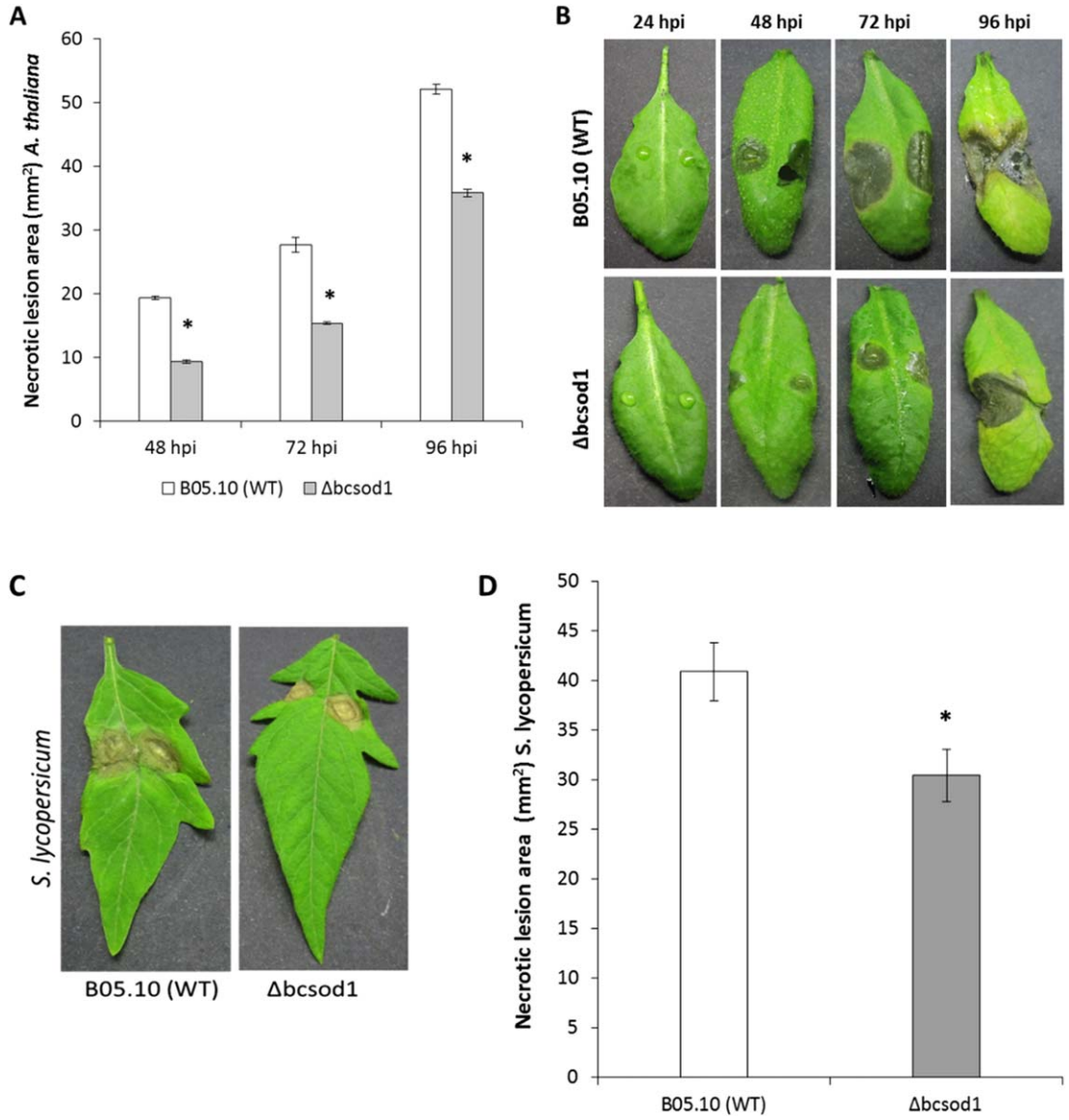


Fig. 1 *Arabidopsis* and tomato plants infected with Δ*bcsod1* or B05.10 (wild-type, WT). (A) Quantification of the necrotic lesion area measured at 48, 72 and 96 h post-inoculation (hpi) in the leaves of *A. thaliana*. (B) Representative photographs of symptoms at 24, 48, 72 and 96 hpi of B05.10 (WT) and Δ*bcsod1* strains in the leaves of *A. thaliana* plants. (C) Representative photographs of symptoms at 72 hpi of B05.10 (WT) and Δ*bcsod1* strains in the leaves of *Solanum lycopersicum* plants. (D) Quantification of the necrotic lesion area measured at 72 hpi in the leaves of *S. lycopersicum* plants. Bars are the means of three independent experiments ± standard deviation (SD) ($n = 18$). Asterisks indicate statistically significant differences ($P < 0.05$, unpaired *t*-test, two-tailed).

Previously, this experimental approach has demonstrated the role of the thioredoxin system in the pathogenesis of *B. cinerea* (Viefhues *et al.*, 2014).

The addition of the antioxidant ascorbic acid (AA) to the spore suspension prior to inoculation decreased the infection rate of B05.10, as expected, but did not rescue the WT infection phenotype in Δ*bcsod1* (Fig. 3C,D). Under these conditions, ROS accumulation was reduced significantly, demonstrating its contribution to the infection process. The absence of infection in the presence of dithiothreitol (DTT) confirms that ROS are crucial in this plant–pathogen interaction (Fig. 3D). Even in the reducing environment of ascorbate, Δ*bcsod1* infection still produced a ROS imbalance in relation to B05.10, with increased superoxide accumulation and reduced peroxide (Fig. 3E–G).

Lack of *bcsod1* increases pathogen-induced callose accumulation

Callose is a plant polysaccharide which is considered to be a good marker of the stress response and a physical barrier against

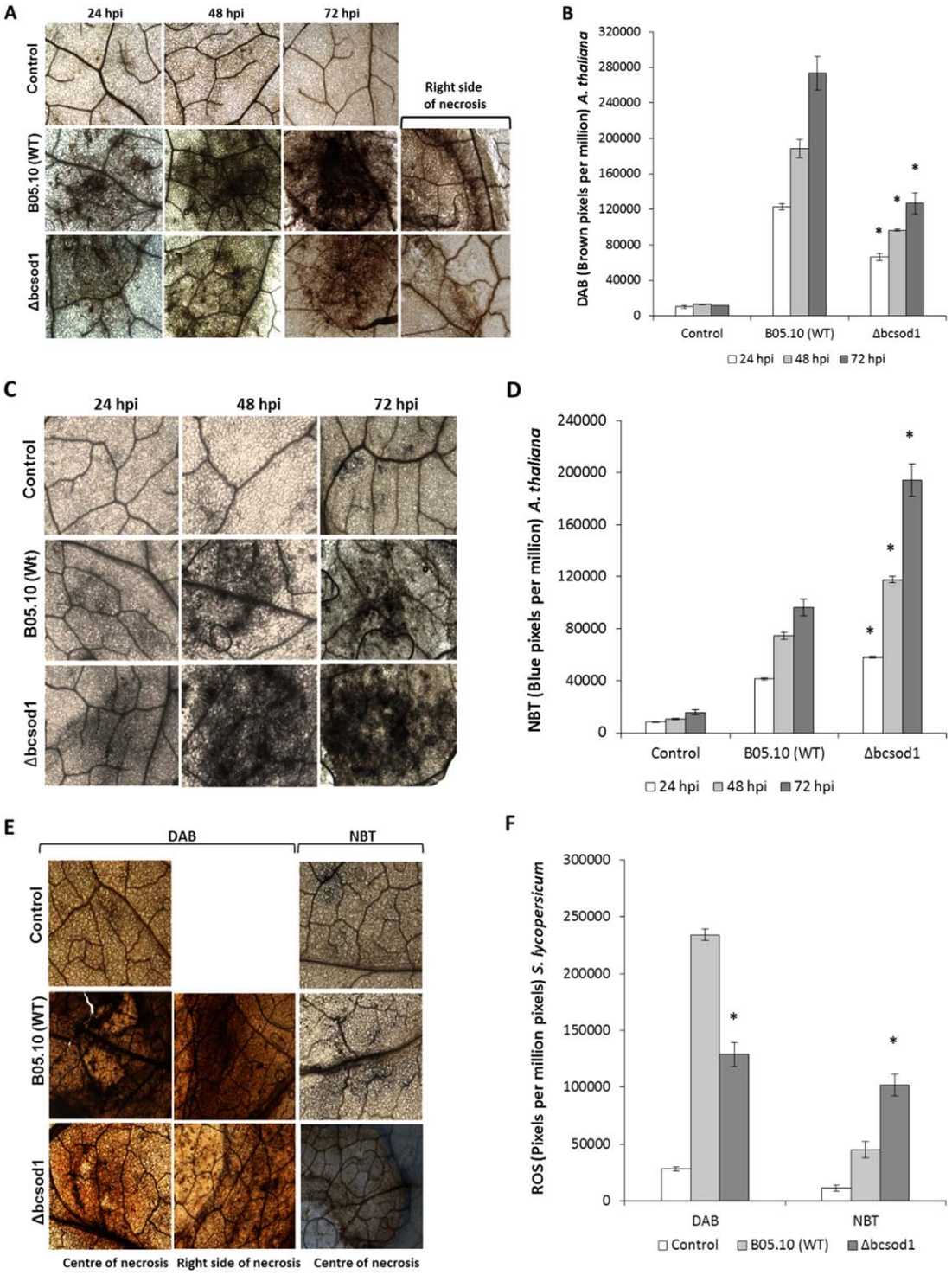


Fig. 2 Hydrogen peroxide (H_2O_2) and superoxide ion (O_2^-) accumulation in *Arabidopsis* and tomato plants inoculated with Δ*bcsod1* or B05.10 (wild-type, WT). The production of H_2O_2 and O_2^- was viewed by 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively. (A) Representative photographs of H_2O_2 accumulation by DAB staining in Arabidopsis leaves at 24, 48 and 72 h post-inoculation (hpi). (B) Quantification of H_2O_2 by digital image analysis at each time point. (C) Representative photographs of O_2^- accumulation by NBT staining in Arabidopsis leaves at 24, 48 and 72 hpi. (D) Quantification of O_2^- by digital image analysis at each time point. (E, F) Representative photographs and quantification of both H_2O_2 and O_2^- accumulation by DAB and NBT staining, respectively, at 72 hpi in tomato leaves. The average of the brown (H_2O_2) or blue (O_2^-) pixels versus the total pixels of 10 photographs per plant was calculated. ROS, reactive oxygen species. The data shown are the means of three independent experiments ± standard deviation (SD) ($n = 18$). Asterisks indicate statistically significant differences ($P < 0.05$, unpaired *t*-test, two-tailed).

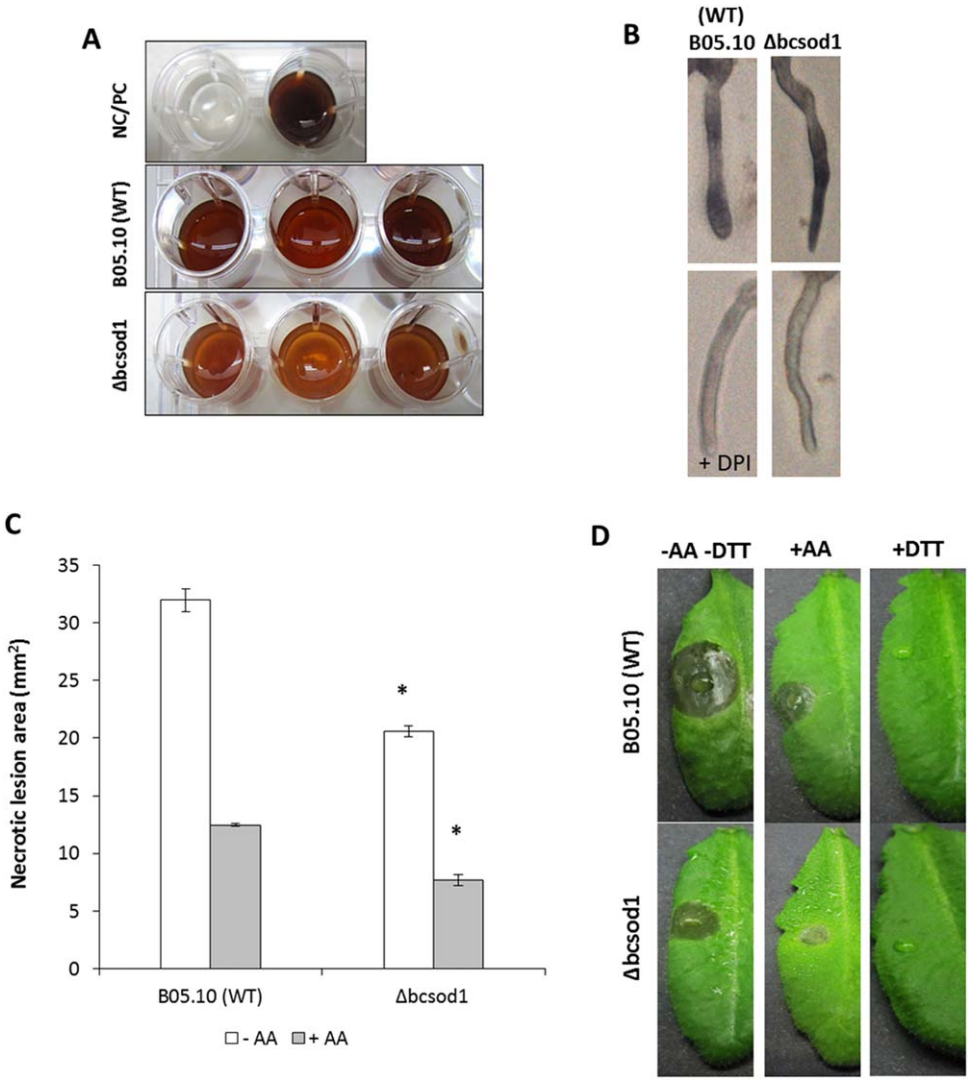


Fig. 3 Hydrogen peroxide (H_2O_2) and superoxide ion (O_2^-) production and influence of the antioxidants ascorbic acid (AA) and dithiothreitol (DTT) on Arabidopsis plants inoculated with Δ*bcsod1* or B05.10 (wild-type, WT). (A) 3,3′-Diaminobenzidine (DAB) test in a microwell plate to analyse H_2O_2 production of both Δ*bcsod1* and B05.10 (WT). Twenty-five milligrams of fresh mycelium were weighed and 1 mL of DAB solution was added. Incubation took place for 2 h in the dark. NC, negative control, DAB solution + 1 μL H_2O_2; PC, positive control, DAB solution + 1 μL H_2O_2 + 1 μL horseradish peroxidase. (B) Superoxide detection in germinated spores (suspension of 10^5 spores/mL) of *Botrytis cinerea* Δ*bcsod1* and B05.10 (WT) by nitroblue tetrazolium (NBT) staining. As a control, diphenyleneiodonium (DPI) (50 μM) was added prior to NBT supplementation. (C) *In planta* infection assay with AA of Δ*bcsod1* and WT. Conidial suspensions of 10^5 spores/mL were supplemented with 5 g of AA per litre prior to inoculation. Quantification of the necrotic lesion area at 72 h post-inoculation (hpi). (D) Representative photographs of the infection assay in Arabidopsis plants with AA and DTT at 72 hpi. Conidial suspensions of 10^5 spores/mL were supplemented with 750 μM of DTT prior to infection. (E) Representative photographs of both H_2O_2 and O_2^- accumulation by DAB and NBT staining, respectively, at 72 hpi in Arabidopsis leaves inoculated with spores supplemented with AA at 72 hpi. (F, G) Quantification of H_2O_2 and O_2^- by digital image analysis in Arabidopsis leaves inoculated with spores supplemented with AA at 72 hpi. The data shown are the means of three independent experiments ± standard deviation (SD) ($n = 18$). Asterisks indicate statistically significant differences ($P < 0.05$, unpaired *t*-test, two-tailed).

several pathogens (Ellinger *et al.*, 2013; Hauck *et al.*, 2003; Vicedo *et al.*, 2009). Aniline blue staining demonstrated increased fungal-induced callose accumulation in Δ*bcsod1*-infected Arabidopsis plants at 72 hpi compared with the plants infected with B05.10 (WT) (Fig. 4A, I). Callose deposits showed a central core region in which most of the polymer accumulated, surrounded by a layer of callose on the edge of the lesions (Fig. 4A, II/III). When we compared Δ*bcsod1* and B05.10 infection, we determined an evident increase in callose on the border, but also in the core region at 72 hpi (Fig. 4A, III). Tomato plants infected with Δ*bcsod1* also showed increased pathogen-induced callose compared with plants infected with WT (Fig. 4B, I). Polymer deposits showed a central

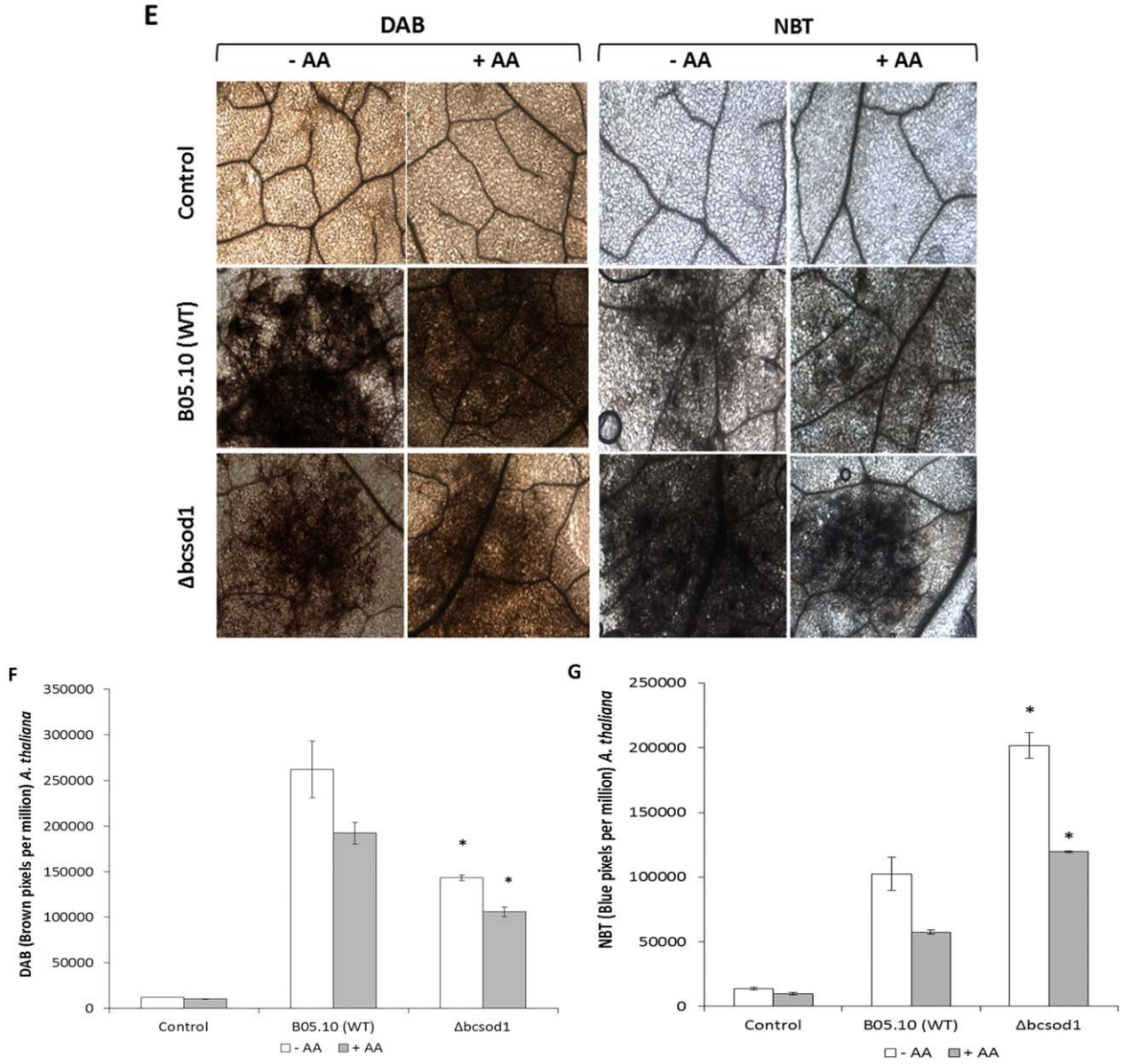


Fig. 3 Continued.

core region in the plants infected by both WT and Δ*bcsod1* in which most of the callose accumulated (Fig. 4B, II/III). In the case of Δ*bcsod1*, the core delimited by a layer of callose was surrounded by a dot-shaped deposit on the border of the infection site instead of the layer observed in B05.10-infected plants (Fig. 4B, II). The different patterns of pathogen-induced callose deposition in the two cultures could be related to the higher susceptibility to B05.10 observed in tomato plants.

To verify the connection between the absence of *bcsod1* and callose metabolism, we analysed the expression of the *PMR4* gene by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). In both Arabidopsis and tomato plants, the transcript levels of the *PMR4* gene were induced significantly following fungal (B05.10) inoculation at all the analysed time points, showing a significant increase at 24 hpi in Δ*bcsod1* mutant-infected plants (Fig. 4A,B, IV).

Changes in metabolite accumulation and gene expression of plants challenged with the Δ*bcsod1* mutant

To determine the impact of the absence of *bcsod1* on the plant basal response, we analysed the accumulation of the main phytohormones and phenolic compounds of Arabidopsis and tomato

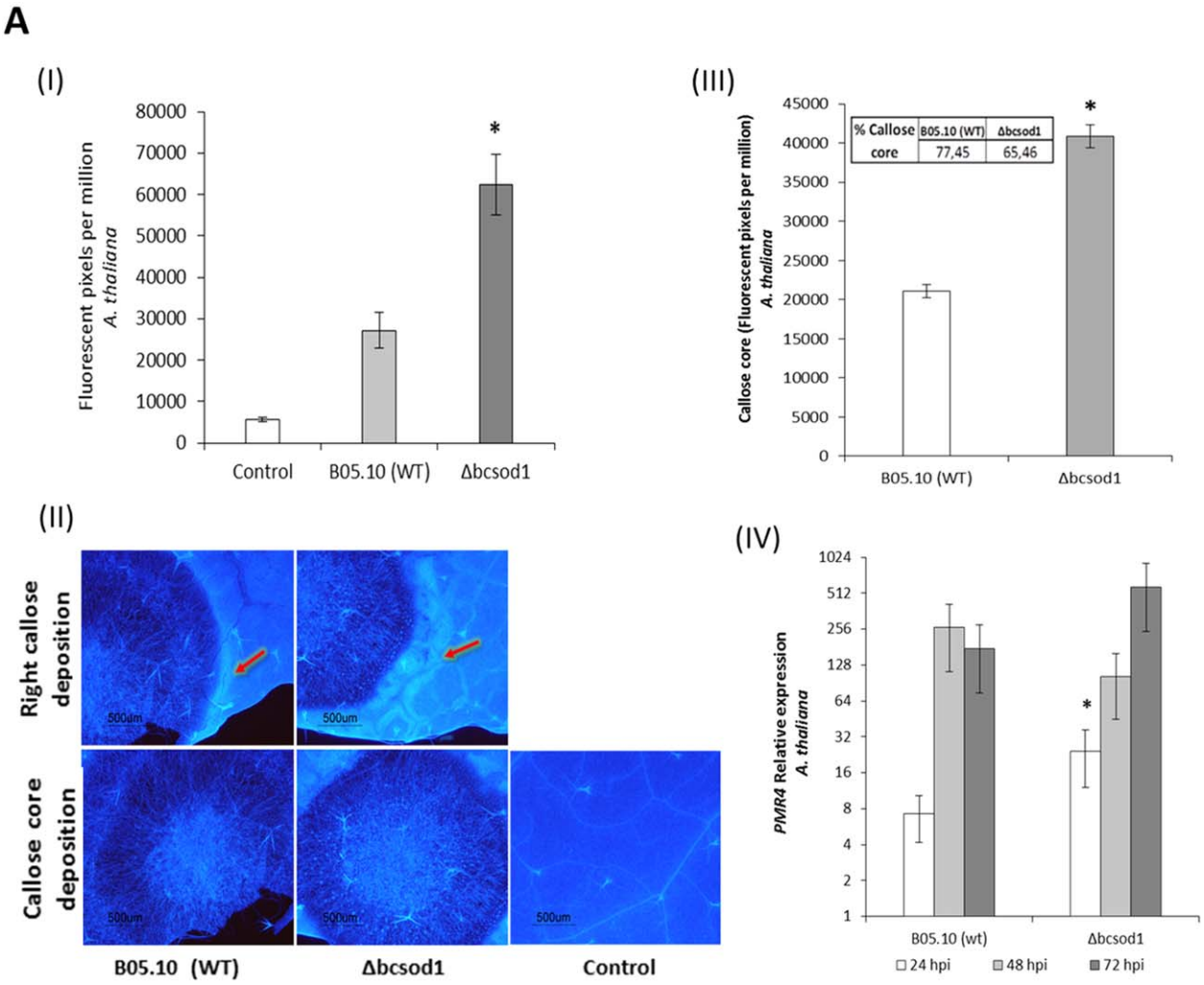
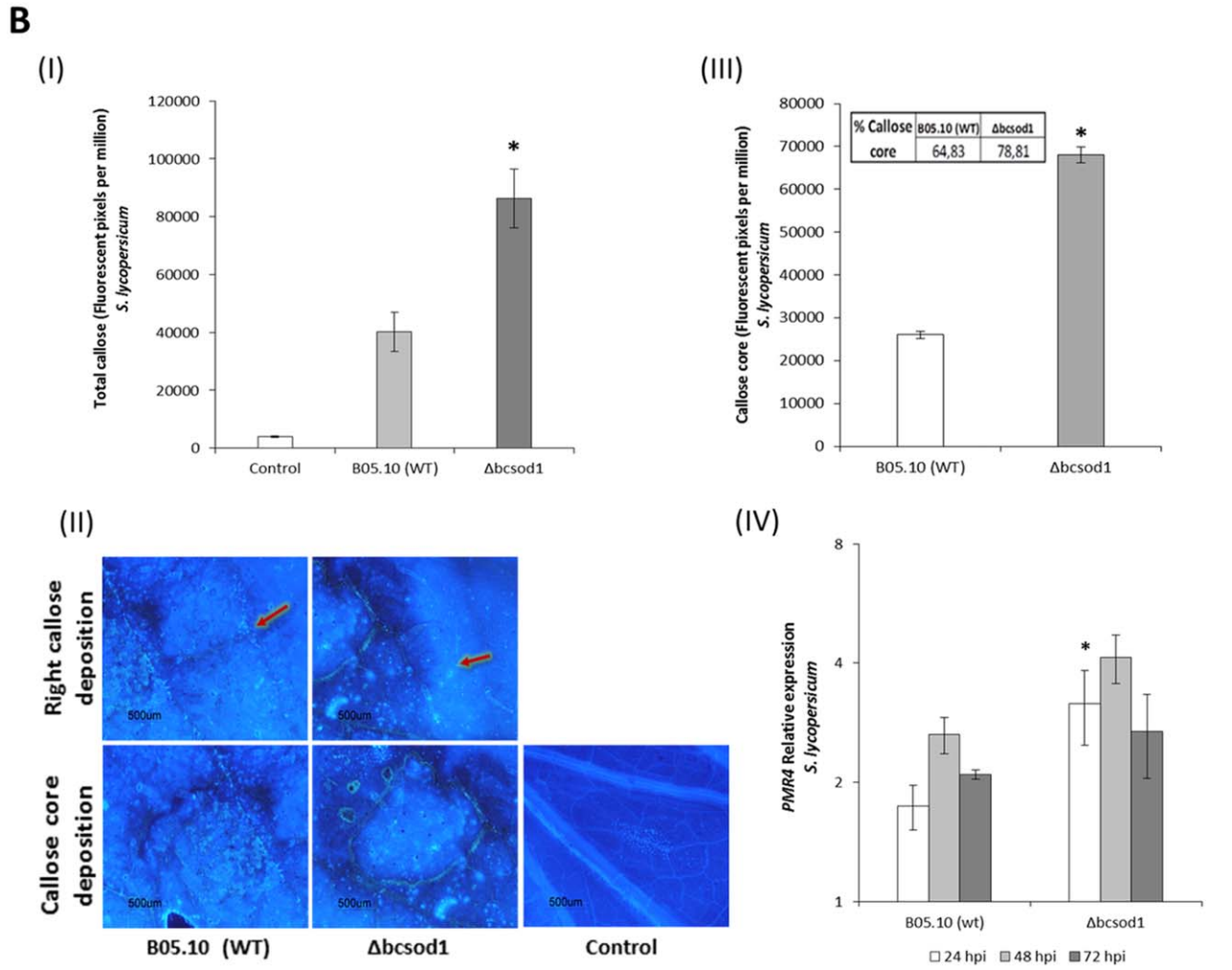


Fig. 4 Callose deposition and *PMR4* expression in Arabidopsis and tomato plants at 72 h post-inoculation (hpi) with Δ*bcsod1* or B05.10 (wild-type, WT). (A, B) I, Quantification of callose visualized by aniline blue staining, performed by the determination of the number of fluorescent pixels per million pixels corresponding to pathogen-induced callose on digital photographs of infected leaf areas. II, Representative photographs of callose staining at 72 hpi with *bcsod1* or B05.10 in *Arabidopsis* and tomato leaves. Arrows indicate deposition of callose on the edge of the lesion. III, Quantification of callose core deposition in the infection area by digital image analysis in Arabidopsis and tomato leaves. Table shows the percentage of callose deposition in the core relative to the total deposition. IV, Relative expression of the callose gene *PMR4* at 24, 48 and 72 hpi. Values presented are the means of three independent experiments ± standard deviation (SD) ($n = 18$). Asterisks indicate statistically significant differences ($P < 0.05$, unpaired *t*-test, two-tailed).

plants infected with the Δ*bcsod1* mutant and B05.10 (WT) (Fig. 5A–G). The analysis was performed by high-performance liquid chromatography (HPLC) coupled to a mass spectrometer and consisted of the simultaneous determination of the following compounds in the same sample [abscisic acid (ABA), SA, jasmonic acid-isoleucine (JA-Ile), JA, OPDA, caffeic acid and ferulic acid] at the following time points (0, 24, 48 and 72 hpi) after infection with B05.10 or Δ*bcsod1* and without infection (mock).

The JA-Ile content increased at 48 hpi in Arabidopsis plants infected with the Δ*bcsod1* mutant compared with those inoculated with B05.10 (Fig. 5). These plants also presented a significant increase in ABA and OPDA at 72 hpi, together with decreased free SA content, compared with those infected with WT (Fig. 5). Interestingly, caffeic acid accumulated rapidly on Δ*bcsod1* mutant infection (24 hpi) and remained stable up to 72 hpi (Fig. 5). This acid is a precursor of lignin and is a component of the papilla (Soylu *et al.*, 2005; Vicedo *et al.*, 2009). The analysis of tomato plants infected with the Δ*bcsod1* mutant showed a similar alteration of the hormone and metabolite profile with a few differences (Fig. 6A–G). As in Arabidopsis, the OPDA and ABA content increased significantly at 72 hpi and the amount of SA decreased at this time point. However, the JA-Ile content remained



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unchanged and the increase in ferulic acid was delayed in tomato plants on Δ*bcsod1* infection compared with plants infected with B05.10 at 72 hpi (Fig. 6).

To verify the connection between the absence of *bcsod1* and the activation of signalling pathways, we analysed the expression patterns of the marker genes for SA (*ICS1*, *PR1*, *PR2*), JA/ET (*PDF1.2*), JA (*VSP1*) and OPDA (*HSP17.4*, *HsfA2*) by RT-qPCR in Arabidopsis plants infected with Δ*bcsod1* and B05.10 at 24, 48 and 72 hpi (Fig. 7A–J).

As expected, B05.10 infection significantly induced *PDF1.2* expression, a resistance marker to necrotrophs, at all time points in Arabidopsis plants. Infection with Δ*bcsod1* remarkably increased *PDF1.2* expression (Fig. 7) at 24 hpi, which correlated with the activation of the JA signalling pathway and reduced virulence of the mutant. The remarkable increase in OPDA accumulation on Δ*bcsod1* infection correlated with the early induction at 24 hpi of the two genes regulated by this oxylipin: *HSP17.4* and *HsfA2* (Masuda *et al.*, 2014; Sham *et al.*, 2014) (Fig. 7). *ICS1* expression increased slightly at 72 hpi in plants infected with Δ*bcsod1* compared with WT, despite the slight reduction observed in free SA levels (Fig. 7). B05.10 infection significantly induced *PR1* expression in Arabidopsis, with similar observations in Δ*bcsod1*-infected plants (Fig. 7). However, *PR2* expression increased in plants infected with Δ*bcsod1* at 24 and 72 hpi compared with those inoculated with B05.10 (Fig. 7).

We also analysed the marker genes for SA (*PR1*, *PR2*), JA (*LoxD*), other oxylipins (*DES*) and OPDA (*HsfA2*) by RT-qPCR in tomato plants infected with Δ*bcsod1* and B05.10 at 24, 48 and 72 hpi (Fig. 8A–H). The absence of the *bcsod1* gene significantly reduced *LoxD* expression, a gene marker of JA signalling, at 72 hpi, but increased *DES* accumulation at this time point, which is involved in other oxylipin synthesis (Itoh and Howe, 2001). The expression of *HsfA2*, a marker gene of OPDA in tomato, increased at all time points in plants challenged with Δ*bcsod1* versus those inoculated with WT (Fig. 8). These changes at the transcriptional level correlate with significant

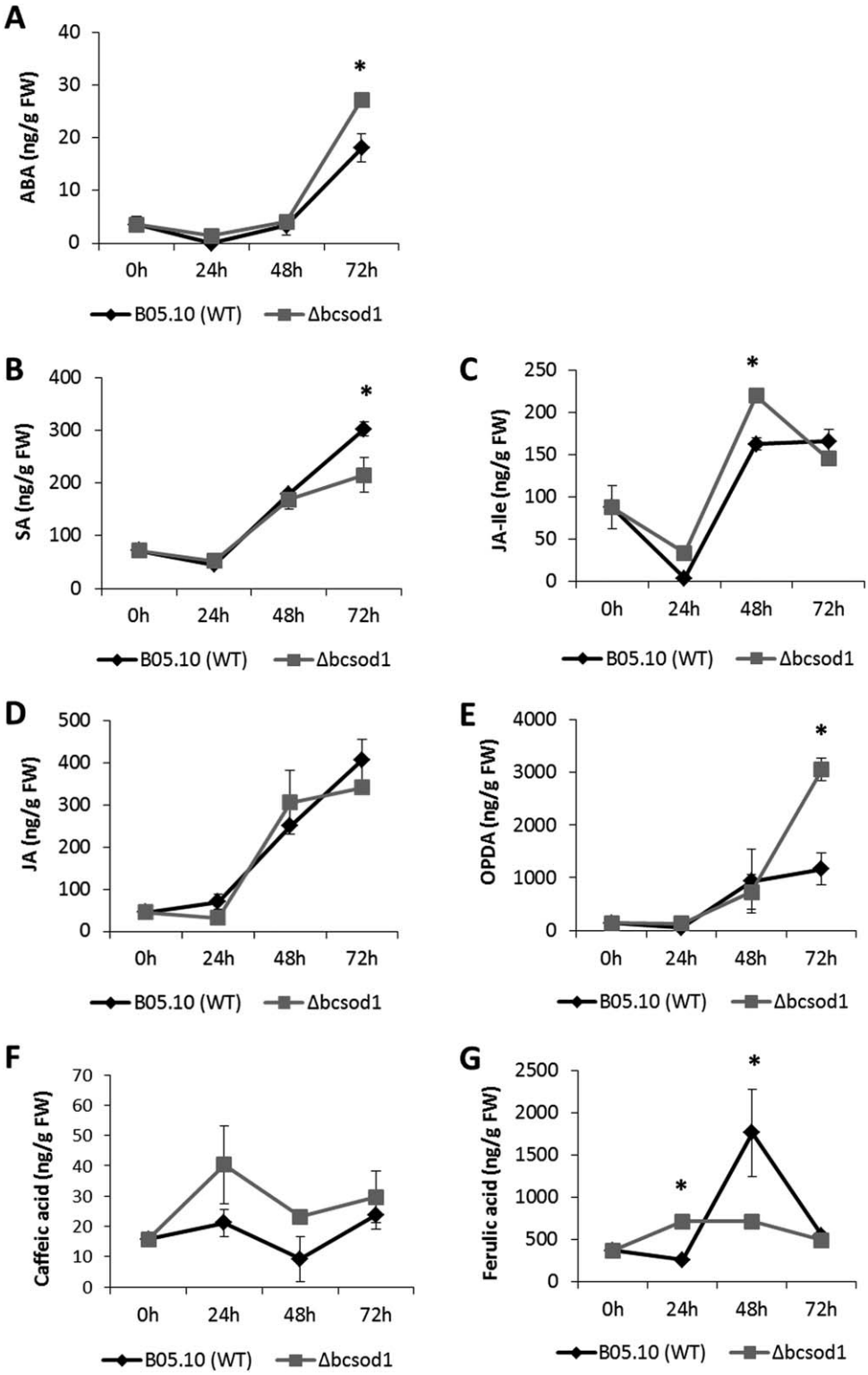

Fig. 5 Hormone and metabolite analysis of Arabidopsis plants not inoculated (mock) and inoculated with Δ*bcsod1* or B05.10 (wild-type, WT). (A–G) Abscisic acid (ABA), salicylic acid (SA), jasmonic acid-isoleucine (JA-Ile), jasmonic acid (JA), 12-oxo-phytodienoic acid (OPDA), caffeic acid and ferulic acid levels determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS) in leaves collected at 0, 24, 48 and 72 h post-inoculation (hpi). Bars are the means of three independent experiments ± standard deviation (SD) ($n = 12$). Asterisks indicate statistically significant differences between plants infected with B05.10 (WT) and Δ*bcsod1* strains at each time point ($P < 0.05$, unpaired *t*-test, two-tailed).

OPDA accumulation and unaltered accumulation of JA-Ile on Δ*bcsod1* infection. In tomato, *PR1* expression increased significantly in response to *Botrytis*, as reported previously (Finiti *et al.*, 2014; Vicedo *et al.*, 2009), and was reinforced in plants infected with Δ*bcsod1* at 72 hpi (Fig. 8). *PR2*, however, decreased slightly (Fig. 8), which indicates that the SA-dependent pathway is also altered in tomato on infection with Δ*bcsod1*, but no changes in free SA accumulation were found.

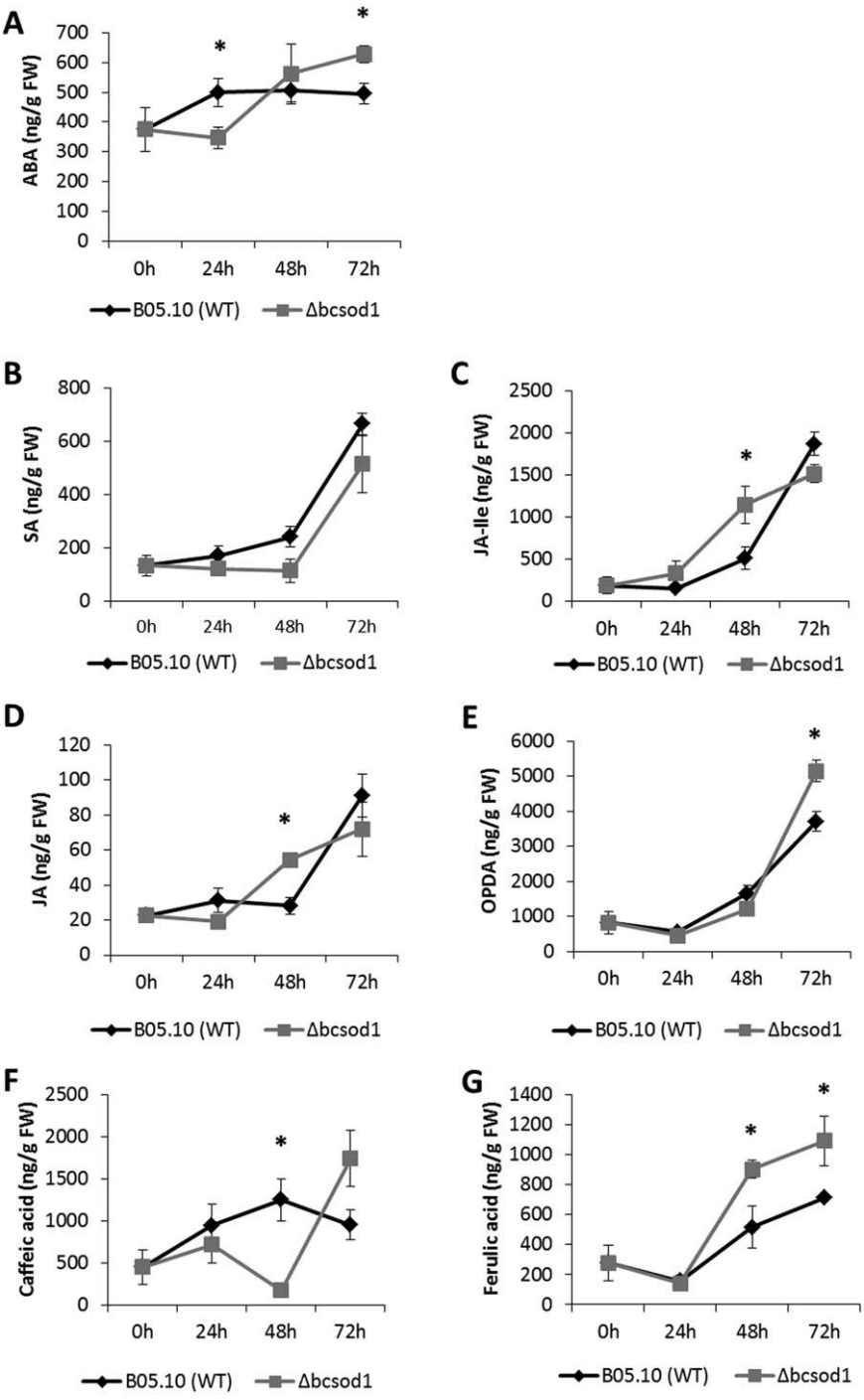

Fig. 6 Hormone and metabolite analysis of tomato plants not inoculated (mock) and inoculated with Δ*bcsod1* or B05.10 (wild-type, WT). (A–G) Abscisic acid (ABA), salicylic acid (SA), jasmonic acid-isoleucine (JA-Ile), jasmonic acid (JA), 12-oxo-phytodienoic acid (OPDA), caffeic acid and ferulic acid levels determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS) in leaves collected at 0, 24, 48 and 72 h post-inoculation (hpi). Bars are the means of three independent experiments ± standard deviation (SD) ($n = 12$). Asterisks indicate statistically significant differences between plants infected with B05.10 (WT) and Δ*bcsod1* strains at each time point ($P < 0.05$, unpaired *t*-test, two-tailed).

We also analysed the expression of the genes related to oxidative stress. No changes in *GST6*, a gene related to ROS detoxification, occurred in Arabidopsis and tomato plants infected with Δ*bcsod1* compared with B05.10 (Figs 7 and 8). However, *WRKY53* and *WRKY33*, which encode transcription factors regulated by oxidative stress, showed a differential expression in plants challenged with *Botrytis* that lacked *bcsod1*. *WRKY53* increased significantly at 48 and 72 hpi in Arabidopsis, but no changes in WRKY33 occurred (Fig. 7), whereas both transcripts were reduced significantly in tomato plants inoculated with Δ*bcsod1* (Fig. 8).

DISCUSSION

The oxidative burst and ROS accumulation are critical factors in *B. cinerea* infection, but the contribution of ROS to the plant–

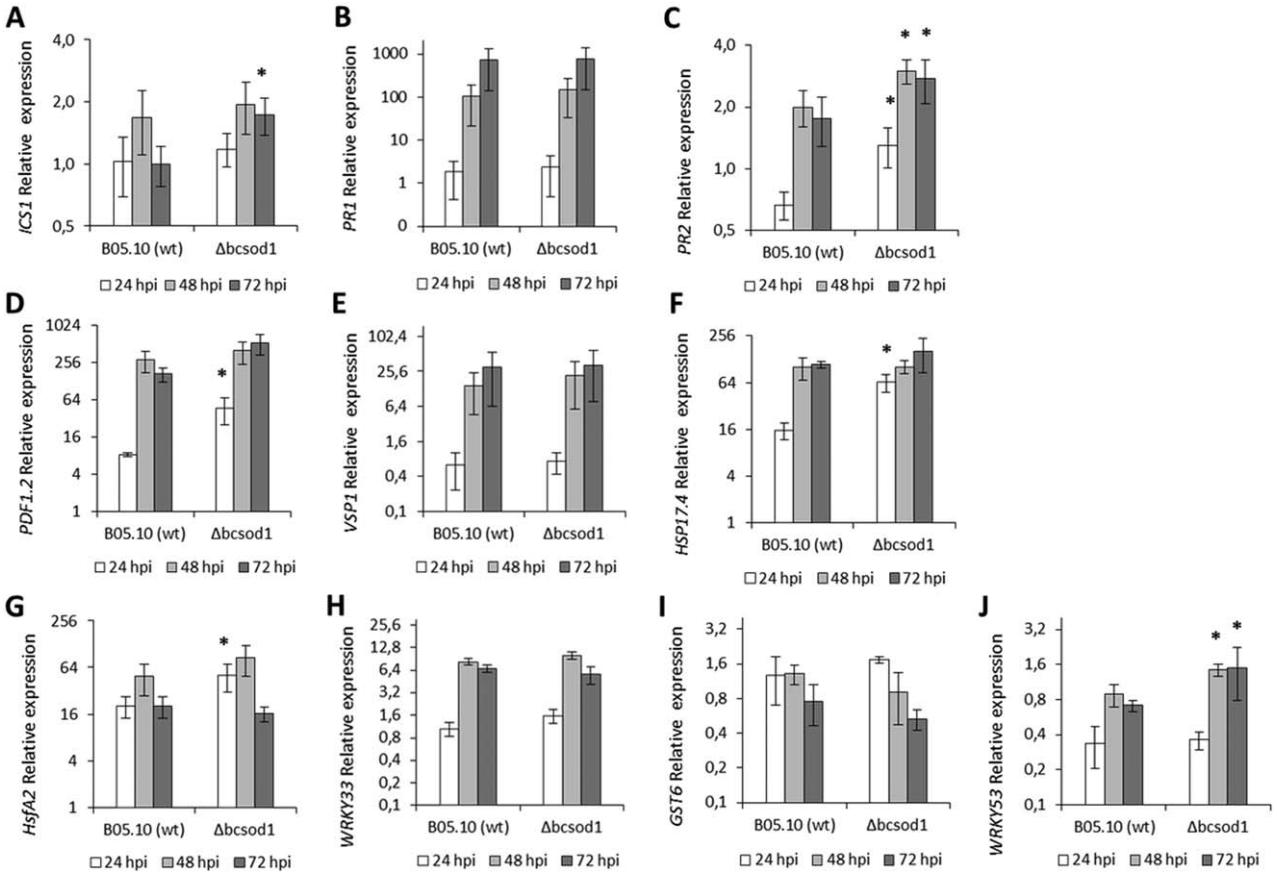

Fig. 7 Expression of the genes involved in signalling and in response to oxidative stress in Arabidopsis plants inoculated with Δ*bcsod1* or B05.10 (wild-type, WT). The relative expression of genes *ICS1*, *PR1*, *PR2*, *PDF1.2*, *VSP1*, *HSP17.4*, *HsfA2*, *WRKY33*, *GST6* and *WRKY53* was determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) in Arabidopsis plants infected with Δ*bcsod1* or B05.10 at 24, 48 and 72 h post-inoculation (hpi) (A–J). *EIF4A1* was used as an internal standard. Data are shown as the $\log_2$ fold change. Bars are the means of three independent experiments ± standard deviation (SD). Asterisks indicate statistically significant differences between plants infected with B05.10 (WT) and Δ*bcsod1* strains at each time point ($P < 0.05$, unpaired *t*-test, two-tailed).

pathogen interaction is complex (Heller and Tudzynski, 2011). Both the plant and pathogen actively produce ROS during infection. ROS play a protective role and act as signalling compounds in plants (Muckenschnabel *et al.*, 2001). Oxidative imbalance forms part of the plant defence response, but *B. cinerea* is also able to stimulate this mechanism for its own benefit (Finiti *et al.*, 2014; Temme and Tudzynski, 2009).

In this article, we have demonstrated that the Δ*bcsod1* mutant shows significantly impaired virulence in Arabidopsis and tomato plants, as reported previously in French bean plants (Patel *et al.*, 2008; Rolke *et al.*, 2004). H_2O_2 accumulation decreased in Arabidopsis plants inoculated with Δ*bcsod1* versus WT (B05.10), whereas superoxide ion (O_2^-) increased. The same alteration in ROS accumulation occurred in tomato plants inoculated with Δ*bcsod1*. Reduced H_2O_2 together with increased O_2^- corresponds to a lack of SOD activity in the Δ*bcsod1* mutant, which catalyses the conversion of O_2^- into H_2O_2 and H_2O. The reduced virulence and observed ROS imbalance could be caused by the inability of Δ*bcsod1* to detoxify endogenous ROS and/or ROS from the host's oxidative burst in response to fungal infection. Although Δ*bcsod1* showed altered ROS production prior to infection, the non-restoration of virulence by antioxidants indicates that *sod1* is not required to cope with the oxidative stress arising during host infection. Hence, BCSOD1 must play another role in the plant–pathogen interaction. The lower infection rate of B05.10 in the presence of a reducing agent supports the notion that a necrotrophic fungus, such as *B. cinerea*, depends largely on direct (or indirect) ROS generation for host tissue colonization (Rolke *et al.*, 2004). The present work supports the contribution of BCSOD1 in the exploitation of the plant oxidative burst for the benefit of the pathogen. BCSOD1 alters the O_2^-/H_2O_2 rate, contributing to the H_2O_2 level, which damages plant tissue and reduces the activation of plant defence, increasing the susceptibility to *B. cinerea*.

Our results indicate that the O_2^-/H_2O_2 imbalance produced in the absence of *bcsod1* contributes to the H_2O_2 level, which signals and facilitates an efficient plant defence, which increases

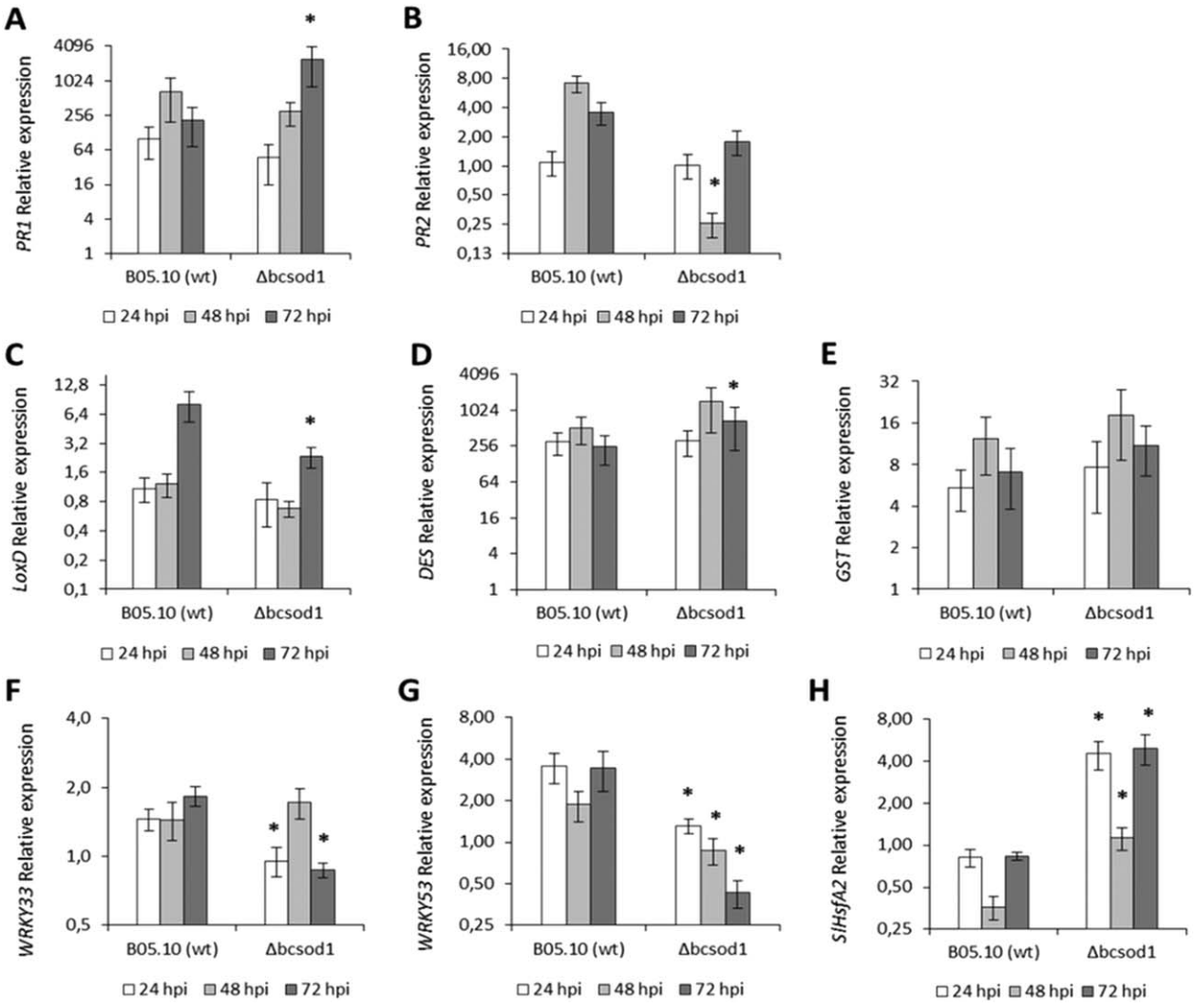

Fig. 8 Expression of the genes involved in signalling and in response to oxidative stress in tomato plants inoculated with Δ*bcsod1* or B05.10 (wild-type, WT). The relative expression of genes *PR1*, *PR2*, *LoxD*, *DES*, *GST*, *WRKY33*, *WRKY53* and *HsfA2* was determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis in tomato plants infected with Δ*bcsod1* or B05.10 at 24, 48 and 72 h post-inoculation (hpi) (A–H). *EF1-α* was used as an internal standard. Data are shown as the $\log_2$ fold change. Bars are the means of three independent experiments ± standard deviation (SD). Asterisks indicate statistically significant differences between plants infected with B05.10 (WT) and Δ*bcsod1* strains at each time point ($P < 0.05$, unpaired *t*-test, two-tailed).

resistance to *B. cinerea*. Hence, BCSOD1 constitutes a virulence factor. Some authors have suggested that the success or failure of certain pathogens depends on H_2O_2 accumulation (Govrin and Levine, 2000; Hückelhoven and Kogel, 2003; Kumar *et al.*, 2001). Our previous data in tomato plants have shown a correlation between H_2O_2 accumulation and *Botrytis* susceptibility, which reflects the importance of the control of ROS accumulation in response to biotic stresses (Angulo *et al.*, 2015; Finiti *et al.*, 2014). The activation of more effective plant defences in the absence of *sod1* supports this hypothesis. Heightened callose deposition occurs in plants following infection with Δ*bcsod1*, depending on the transcriptional regulation of callose synthase. These data demonstrate the connection between changes in the O_2^-/H_2O_2 ratio and defensive polysaccharide metabolism. A similar connection was shown by Vellosillo *et al.* (2010), where O_2^- and singlet oxygen ($^1O_2$) induced a stronger accumulation of callose than did H_2O_2. We have reported previously the importance of callose deposition in the plant response to *B. cinerea* (Finiti *et al.*, 2013; Flors *et al.*, 2007; Vicedo *et al.*, 2009). In Δ*bcsod1*-infected Arabidopsis and tomato plants, the callose core at the infection site increased. This callose core in the infection area was observed in Arabidopsis plants that overexpressed the callose synthase gene *PMR4*, leading to increased resistance to the adapted powdery mildew *Golovinomyces cichoracearum* and the non-adapted powdery mildew *Blumeria graminis* f. sp. *hordei* (Naumann *et al.*, 2013). Recently, it has been proposed that callose itself is likely to act

antagonistically on SA defence signalling, which is suggestive of the role of *PR2* as a modulator of callose- and SA-dependent defence responses (Oide *et al.*, 2013). These authors showed that ABA promotes callose deposition through the transcriptional repression of *PR2* in Arabidopsis challenged with *Leptosphaeria maculans* and *Pseudomonas syringae*. In our experimental system, callose acted as an antagonist of SA signalling in Arabidopsis and tomato plants. At the same time, the different regulation of *PR2* in both cultures could explain the distinct pattern of callose deposition on the border of the infection site in response to Δ*bcsod1*. This could also be related to the higher susceptibility to B05.10 shown by tomato plants compared with Arabidopsis.

The absence of *bcsod1* profoundly altered hormone signalling involved in plant basal responses. In Arabidopsis plants, both JA-Ile and OPDA signalling were activated. OPDA acts in a JA-independent manner by signalling to amino acid biosynthesis and cellular redox homeostasis in stress responses (Park *et al.*, 2013). Sham *et al.* (2014) reported that *Hsp17.4* is one of the genes commonly induced by *B. cinerea* infection, OPDA and oxidative stress in Arabidopsis. In tomato plants, the activation of OPDA signalling and the involvement of other oxylipins in plant protection against oxidative stress was observed, as shown previously (Angulo *et al.*, 2015).

SA signalling was also activated on Δ*bcsod1* infection versus B05.10 in both cultures. This agrees with the concept that the effectiveness of plant responses against pathogens is much more complex than the classical antagonism between the SA and JA/ET pathways (Caarls *et al.*, 2015; Grant and Jones, 2009). This also indicates that plant susceptibility relies on hormonal balance, which may differ depending on the culture. The key role of both callose and OPDA-mediated signalling in the plant response to *Botrytis* is noteworthy. In tomato, the silencing of *OPR3* showed that OPDA alone plays a major role in basal defence against this necrotroph (Scalschi *et al.*, 2015). In Arabidopsis, there is controversy with regard to whether OPDA promotes resistance to *Alternaria brassicicola* in the absence of JA, because experimental conditions could mask the possible contribution of OPDA to pathogen resistance (Schilmiller *et al.*, 2007; Stintzi *et al.*, 2001). Our results strongly support the role of this oxylipin in the defence against *Botrytis* in both species. Previously, it has been shown that the oxylipins, which are formed by singlet oxygen, induce callose deposition (Vellosillo *et al.*, 2010).

Our results also showed changes in *WRKY53* and *WRKY33* in plants infected with Δ*bcsod1* versus B05.10. WRKY transcription factors control diverse processes, such as the response to pathogens or wounding, but also leaf senescence (Rushton *et al.*, 2010). *WRKY53* codes for a transcription factor that acts as a positive regulator of basal resistance, but also as a negative regulator of certain SA responses in Arabidopsis (Murray *et al.*, 2007). Its induction is H_2O_2 dependent in Arabidopsis and tobacco plants (Golemiec *et al.*, 2014; Miao and Zentgraf, 2007), and the expression of a redox-sensitive transcription factor positively regulates age-induced leaf senescence (Xie *et al.*, 2014). This gene is also involved in priming defence responses by epigenetic modifications (Jaskiewicz *et al.*, 2011). In tomato, we have shown previously that the induction of the *WRKY53* gene, which responds to *Botrytis*, is also primed by the natural inducer hexanoic acid on infection (Finiti *et al.*, 2014). Recently, Van Eck *et al.* (2014) have demonstrated that the *WRKY53* transcriptional network regulates oxidative responses to a wide array of stresses in rice.

WRKY33 plays a critical role in plant resistance to necrotrophic pathogens, including *B. cinerea* (Birkenbihl *et al.*, 2012). The role of *WRKY33* in Arabidopsis resistance to necrotrophic pathogens is linked to its positive role in the regulation of genes involved in the JA response and redox homeostasis (Birkenbihl *et al.*, 2012).

In conclusion, although it is difficult to discriminate between fungal and plant contributions to ROS production, our results demonstrate that changes in ROS production, specifically O_2^-/H_2O_2 imbalance, caused by the absence of fungal *bcsod1*, promote efficient defence responses, such as callose deposition, which are orchestrated by complex signalling crosstalk. This supports the hypothesis that SOD is important for the full virulence of *Botrytis* given its ability to produce H_2O_2 and exploit the plant oxidative burst, as suggested previously (Rolke *et al.*, 2004; Tiedemann, 1997). Our data also support the notion that ROS play different roles in plants challenged by this necrotroph in accordance with the dynamics and specificity of ROS signalling and in networks with other signalling pathways, as reported previously in other systems (Mittler *et al.*, 2011). The specific signalling roles of ROS need to be determined.

EXPERIMENTAL PROCEDURES

Plant material, microbial strains and growth conditions

Seeds of *A. thaliana* N70000 (WT) were pretreated at 4 °C in the dark for 48 h to synchronize germination. Plants were grown in Jiffy-7 pots (www.jiffygroup.com) in a growth chamber at 23 °C day/19 °C night, 8 h of light/day and 70% relative humidity (RH) for 5 weeks before being used. The N70000 ecotype was obtained from the Nottingham Arabidopsis Stock Centre (NASC) (http://Arabidopsis.info/). Tomato (*Solanum lycopersicum*) cv. Ailsa Craig plants were grown in commercial peat in a glasshouse with 16 h of daylight. Tomato plants were grown for 4 weeks prior to use.

Botrytis cinerea strain B05.10 (WT) and mutant strain Δ*bcsod1* were kindly provided by Paul Tudzynski (University of Munster, Germany). Both strains were cultured on potato dextrose agar (PDA; Scharlau Microbiology, Sentmenat, Barcelona, Spain, 01-483-500) containing 0.5% sucrose. The Δ*bcsod1* strain was grown in the presence of hygromycin (70 μg/mL). Both strains were grown under dark conditions at 21 °C with exposure to UV-A light (350–400 nm) for 15 min every 3 h.

Botrytis cinerea challenge

The spores of both strains were collected from 7-day-old cultures with sterile water that contained 0.02% (v/v) Tween-20, which was then filtered, quantified with a haemocytometer and adjusted to the appropriate concentration. Prior to infection, spores were incubated in sterile water that contained 50% (v/v) of supplemented Gambor's B5 medium (Sigma-Aldrich, St. Louis, MO, USA, Ref. G5893) for at least 2 h. The spore density for infections in Arabidopsis plants was adjusted to 10^5 spores/mL, and to 5×10^4 spores/mL for infections in tomato plants. All the plants were inoculated with two drops (5 μL) of conidial suspension per leaf (four leaves per plant in Arabidopsis and on the third and fourth leaves of tomato). Disease symptoms were assessed by determining the average necrotic lesion areas in leaves of living plants. All the experiments were performed with at least six plants.

Hormone and metabolite analysis

The extraction and quantification of hormones and metabolites were performed using an HPLC Waters Alliance 2690 (Milford, MA, USA) with a reverse phase column Nucleosil-ODS (100 mm × 2 mm i.d.; 5 μm) (Scharlab, Barcelona, Spain), as described in Vicedo *et al.* (2009). HPLC was coupled to a quadrupole-hexapole-quadrupole Quatro-LC (Micromass, Manchester, UK) mass spectrometer. Samples were obtained from leaves inoculated as described previously in living plants. The leaves at time 0 h and mock leaves were not infected. Quantitative data were processed by the Masslynx NT program, version 3.4 (Micromass; http://www.micromass.co.uk). Three replicate experiments were run with 12 plants per genotype/condition, and similar results were obtained.

ROS quantification

Leaves infected with both strains of *B. cinerea* were collected at 24, 48 and 72 hpi and were incubated with DAB for 8 h to view H_2O_2. For superoxide ion, leaves collected at the same time points were incubated in NBT for 2–3 h. Subsequently, samples were washed with 96% ethanol to remove chlorophyll and unbound stains. Samples were obtained from the leaves inoculated as described previously in living plants. Control leaves were not infected. H_2O_2 and superoxide ion accumulation were quantified with Gimp 2.6 software (http://www.gimp.org), and were expressed as the average number of brown pixels per million pixels and blue–grey pixels per million pixels, respectively, on digital photographs of infected leaf areas. For the production of H_2O_2 on microwell plates, strains were grown on PDA overlaid with cellophane for 3 days; 25 mg of fresh mycelium was weighed and placed in 1 mL of fresh DAB solution (0.5 mg of DAB per millilitre); 8 h after incubation in the dark at room temperature, the resulting coloration was recorded photographically. For the negative control, 1 mL of DAB supplemented with 1 μL of H_2O_2 (30%) was used and, for the positive control, 2 μL of horseradish peroxidase (Sigma-Aldrich, P8375) and 1 μL of H_2O_2 (30%) were added to 1 mL of DAB. For superoxide ion accumulation in the hyphae of both *B. cinerea* strains, drops of 20 μL, which contained 10^5 spores/mL, were placed on the surface of a slide and were germinated in a moist chamber overnight. Samples were stained by applying a drop of 20 μL of NBT (0.05% w/v), and were monitored and photographed by microscopy 2 h after staining. As a control, 20 μL of DPI (50 μM; diphenyleneiodonium chloride, Sigma, St. Louis, MO, USA, D2926) were added to some of the samples. For *in planta* infection assay with AA or DTT of Δ*bcsod1* and WT, conidial suspensions of 10^5 spores/mL were supplemented with 5 g of AA per litre or 750 μM of DTT prior to inoculation. Quantification of the necrotic lesion area was examined at 72 hpi. The assays were repeated three times with at least six plants per genotype/condition, and similar results were obtained.

Callose deposition

Callose was determined by aniline blue staining, as described by Flors *et al.* (2007). Samples were obtained from leaves inoculated as described previously in living plants. Control leaves were not infected. The quantification of infected areas was performed by digital image analysis, and by calculation of the number of fluorescent pixels per million pixels with Gimp 2.6 software. The assay was repeated three times with at least six plants per genotype/condition, and similar results were obtained.

Analysis of gene expression by RT-qPCR

Total RNA was extracted from samples obtained from leaves inoculated as described previously in living plants with the NucleoSpin RNA Plant kit (Macherey-Nagel GmbH, Düren, Germany & Co; Ref. 740949.50). RNA quantification was performed using a NanoDrop ND1000, Thermo Scientific, Wilmington, DE, USA. cDNA was obtained as described by Finiti *et al.* (2013). Relative quantification was used to determine the differential expression of genes. Arabidopsis *EIF4A1* and tomato *EF1-α* were employed as internal reference. The relative expression was determined by the ΔΔCt method (Pfaffl, 2001); data are shown on a logarithmic scale base 2. Real-time efficiency (*E*) was calculated for each primer pair according to the equation: $E = 10[-1/\text{slope}]$. Three replicates were performed with at least six plants per genotype/condition, and similar results were obtained. The genes and primers used for the Arabidopsis and tomato analyses are reported in Table S1 (see Supporting Information).

Statistical analysis

Differences between groups were calculated with Student's *t*-test ($P < 0.05$, unpaired *t*-test, two-tailed). All the experiments were repeated three times with a minimum of six plants for each genotype/condition per experiment. The values shown are the means of the independent biological replicates ± standard deviation (SD).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Primers used for reverse transcription quantitative polymerase chain reaction (RT-qPCR).