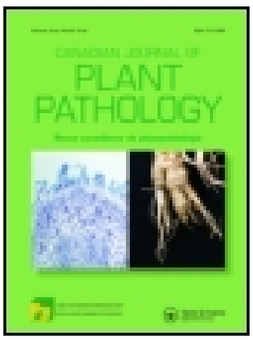

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Role of UV-damaged DNA-binding protein-1 (DDB1) in early response to *Pseudomonas syringae* pv. tomato DC3000 infection in tomato

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Abstract: Diseases caused by pathogens are one of the most important stress factors that affect crop yield and quality and plants have evolved a complex network of responses to infection. Tomato UV-damaged DNA-binding protein-1 (DDB1) has been implicated in playing a role in resistance against *Agrobacterium* infection. However, the effect of DDB1 protein on plant disease resistance is still largely unknown. *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) is an important pathogen of tomato and has been used as a model for testing disease susceptibility. In this work, we compared the sensitivity to *Pst* DC3000 among wild-type seedlings AC^{+}, DDB1-deficient mutants (*hp1*) and DDB1 over-expression (DDB1-OE) seedlings. Results showed that the *hp1* mutant is more susceptible to *Pst* DC3000, whereas the resistance was increased in the DDB1-OE seedlings. Hydrogen peroxide, the key component of the plant defense response and the corresponding cell death phenotype were compromised in the *hp1* mutant and enhanced in the DDB1-OE plants. Consistent with that, salicylic acid production and *PR1a1* gene expression were positively regulated by the *DDB1* gene upon infection with *Pst* DC3000 at 24 h after inoculation. Other pathogenesis-related genes (*Gras2*, *Lrr22*, *Pti5*, *Wrky28* and mitogen-activated protein kinase 3) were also induced in the DDB1-OE plants but compromised to different degrees in the *hp1* mutant. Overall, this study suggests that DDB1 plays a regulatory role in tomato defense responses likely through modulating SA-associated signaling pathways.

Keywords: Arabidopsis, *Solanum lycopericum, Pseudomonas syringae*, immune

response, salicylic acid, defense response.

Résumé : Les maladies causées par des agents pathogènes sont un des plus importants facteurs de stress qui influencent le rendement et la qualité des cultures, et les plantes ont développé un réseau complexe de réactions à l'infection. La protéine-1 liée à l'ADN (DDB1) de la tomate, endommagé par le rayonnement UV, a joué un rôle dans la résistance à l'infection causée par *Agrobacterium*. Toutefois, l'effet de la protéine DDB1 sur la résistance de la plante à la maladie est largement inconnu. *Pseudomonas syringae* pv. tomate DC3000 (*Pst* DC3000) est un agent pathogène important de la tomate et il a été utilisé comme modèle afin de tester la susceptibilité aux maladies. Dans cette étude, nous avons comparé la sensibilité à *Pst* DC3000 de plantules sauvages AC^{+}, de mutants déficients en DDB1 (*hp1*) et de plantules affichant une surexpression de la DDB1 (DDB1-OE). Les résultats ont montré que le mutant *hp1* est plus réceptif à l'égard de *Pst* DC3000, tandis que, chez les plantules DDB1-OE, la résistance était accrue. Le peroxyde d'hydrogène, le composant clé du mécanisme de défense de la plante, et le phénotype de mort cellulaire correspondant ont été compromis chez le mutant *hp1* et accrus chez les plantules DDB1-OE. En concordance à cela, la production d'acide salicylique et l'expression du gène *PR1a1* ont été positivement régulées par le gène de la DDB1 lors de l'infection causée par *Pst* DC3000, 24 heures après l'inoculation. D'autres gènes liés à la pathogenèse (*Gras2*, *Lrr22*, *Pti5*, *Wrky28* et protéine kinase 3 activée par des agents mitogènes) ont aussi été induits dans les plantules DDB1-OE, mais compromis

à différents degrés chez les mutants *hp1*. En général, cette étude suggère que la DDB1 joue un rôle de régulation dans les mécanismes de défense de la tomate, probablement en modulant les voies de signalisation associées à l'acide salicylique.

Mots clés : Arabidopsis, *Solanum lycopericum, Pseudomonas syringae*, réaction immunitaire, acide salicylique, mécanismes de defense.

Introduction

Plants possess multiple defense signaling pathways to recognize and prevent pathogen invasion (Jones & Dangl 2006). Microbe patterns such as flagellin, peptidoglycan, and chitin can induce a basal defense known as pattern triggered immunity (PTI). Pattern recognition receptors mediate microbial pattern recognition and plant immune activation (Albert et al. 2015). This leads to the activation of a series of cellular events to eventually prevent pathogen colonization, including callose deposition in the cell wall, reactive oxygen species (ROS) accumulation, and induction of plant pathogenesis-related gene induction (Segonzac & Zipfel 2011; Kadota et al. 2015). Generally, PTI is effective in preventing the invasion by most microorganisms. However, some pathogens can secrete defense-suppressing effector proteins into the apoplastic space or cytoplasm to overcome PTI (Chisholm et al. 2006). In response, plants have evolved effector-triggered immunity (ETI) to cope with specific effectors released by pathogens (Jones & Dangl 2006; Tsuda et al. 2009). Numerous resistance proteins are expressed to detect the pathogen effectors before a hypersensitive response is induced that leads to plant cell death. Consequently, the pathogen growth is retarded (Jones & Dangl 2006).

It has been suggested that several hormone molecules, including salicylic acid (SA), jasmonic acid (JA), and ethylene regulate the plant immune response. Among them, SA regulates defense responses associated with biotrophic pathogen infection, whereas JA and ethylene mediate resistance against necrotrophic pathogens (Halim et

al. 2009; Yang et al. 2015). The induction of *PR-1* has been confirmed as a marker gene for the SA signaling pathway (Durrant & Dong 2004). *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) has been used as a model pathogen for testing disease susceptibility and hormone signaling transduction in plants, both in its natural host tomato (*Solanum lycopersicum*), but also in *Arabidopsis thaliana* (Xin & He 2013). It is known that SA is vital for promoting disease resistance in response to *Pst* DC3000 (Agorio & Vera 2007). Accumulation of SA at infection sites can diffuse in plant tissues and lead to systemic resistance against *Pst* DC3000 throughout the plant. This phenomenon is called systemic acquired resistance (Durrant & Dong 2004).

Tomato is the one of the most important vegetable crops worldwide and is also an experimentally important crop (Kimura & Sinha 2008). A *high pigment* mutant (*high pigment-1*, *hp1*) with exaggerated photo responsiveness and elevated pigmentation has been described in tomato (Kendrick et al. 1997). The *HIGH P1GMENT-1* gene encodes UV-Damaged DNA-Binding Protein-1 (DDB1) (Liu et al. 2004) that was initially identified as an important component of a UV-induced DNA damage repairing complex. Later, studies revealed that DDB1 is also a component of more than 90 ubiquitin-E3 ligases and functions as a substrate or adapter protein between Cullin 4A (CUL4A) and CUL4-associated factors to target substrates in many cellular processes, including cell cycling (Roodbarkelari et al. 2010), ABA signal transduction (Lee et al. 2010) and epigenetic regulation (Pazhouhandeh et al. 2011). A previous study indicated that the *hp1* mutant is more susceptible to *Agrobacterium tumefaciens* infection compared with the wild-type (Liu et al. 2012a).

However, the role of DDB1 in plant defense responses is still largely unclear. In this study, we inoculated the tomato wild-type 'Ailsa Craig' plus (AC^+), *hp1* mutant and DDB1 over-expression (DDB1-OE) plants with *Pst* DC3000 to identify the function of DDB1 in tomato defense responses.

Materials and methods

Plants and growth conditions

Tomato plants, including AC^+, *hp1* mutant, and DDB1-OE were used in this study. The DDB1-OE plant was constructed previously (Liu et al. 2012b) and had been identified as homozygous. All plants were grown in a greenhouse under diffused daylight and temperature range of 22-26°C with 16 h light (100 μmol m^{-2} s^{-1}) and 8 hr dark.

Pathogen inoculation

Pst DC3000 (virulent) was cultured at 28°C in King's B medium (Zhao et al. 2013) for 48 h. Cells were harvested by centrifugation at 5000×g for 5 min, resuspended to 1×10^8 colony-forming units (cfu) mL^{-1} in 10 mM $MgCl_2$ with 0.006% Silwet L-77. Eight-week-old plants were sprayed with the suspension and incubated at 26°C in an incubator (MLR-352H, SANYO, Japan) with a relative humidity of 90%. The plants treated with 10 mM $MgCl_2$ served as the mock inoculation.

Bacterial growth assay

About 0.2 g of leaf samples were collected at 1, 3, 5, 7 days post-inoculation (dpi) to determine cfu mL^{-1} of *Pst* DC3000. Samples were accurately weighed and homogenized in 2 mL of 10 mM $MgCl_2$. The suspension was diluted and plated on King's B medium containing 50 μg mL^{-1} of rifampicin. The plates were kept at 28°C for 2 days and the number of bacteria per gram fresh weight (cfu g^{-1} FW) was counted. Each treatment contained three replicates with five plants per replicate; the experiment was repeated three times.

Chemical treatments

Leaf samples were collected at 48 h post inoculation (hpi) for hydrogen peroxide (H_2O_2) detection and dead cell staining. The accumulation of H_2O_2 in AC^+, *hp1* mutant and DDB1-OE plants was detected by 3,3′-diaminobenzidine (DAB) staining as described by Thordal-Christensen et al. (1997). Trypan blue staining was performed to detect the lesion sites using lactic acid-phenol-trypan blue solution as described by Bowling et al. (1997). Each treatment contained three replicates with five plants per replicate; and the experiment was repeated three times.

SA content determination and gene expression analysis by qRT-PCR

For SA content determination, leaf samples were collected at 24 hpi. Free SA accumulation was extracted and analyzed by HPLC according to López-Gresa et al. (2016); plants treated with 10 mM $MgCl_2$ served as the mock inoculation group. For gene expression analysis, leaf samples were collected at 0, 6, 24 and 48 hpi. Total mRNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the

supplier's instructions. About 100 ng of mRNA was used for cDNA synthesis. Real-time qRT-PCR was performed using the cDNA; the gene-specific primers used are presented in Table 1. A Step ONE Plus Real-Time PCR System (Applied Biosystems) was applied for the qRT-PCR. PCR was performed using SYBR Green PCR Master Mix (Transgen, Beijing, China). The PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 15 s. A melting curve analysis of amplification products was performed at the end of the PCR reaction. The melting cycle was 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The *UBI3* gene was used as the internal control, using the $2^{-\Delta\Delta CT}$ method according to Livak & Schmittgen (2001). Each treatment contained three replicates with 5 plants per replicate, and the experiment was repeated three times.

Statistical analysis

All statistical analyses were performed using SAS 9.2 (Cary, NC). Data were analyzed by one-way analysis of variance. Mean separations were performed by Duncan's multiple range tests. Least square means (LSMEANS) and standard errors of least-squares means (SEM) were calculated using the LSMEANS. Differences at $p<0.05$ were considered significant.

Results and discussion

Effect of DDB1 on disease resistance

To test whether DDB1 contributes to the basal defense against virulent pathogens,

AC^+, *hp1*, and DDB1-OE plants were inoculated with *Pst* DC3000. Three days after inoculation, plants began to develop disease symptoms; these were visibly more severe on the *hp1* mutant compared with AC^+ or DDB1-OE. At 7 dpi, there were more spots on *hp1* leaves than on AC^+. Conversely, symptoms on DDB1-OE plants were relatively moderate (Fig. 1A). Additionally, the bacteria growth assessment showed that the bacteria number increased gradually in all plants tested within 7 dpi (Fig. 1B) and was higher in the *hp1* mutant ($P<0.05$) and lower in DDB1-OE compared with AC^+ ($P<0.05$).

In a previous study, the *hp1* mutant was observed to be more susceptible to *A. tumefaciens* GV2260 and EHA105 than the wild-type AC^+ (Liu et al. 2012a), consistent with our results using *Pst* DC3000. The slower bacterial growth and milder symptoms on DDB1-OE plants suggested that DDB1 was required for plant resistance against bacterial infection. The loss of function of DDB1 facilitated the *Pst* DC3000 infection, while over-expression of DDB1 contributed to the increased level of disease resistance.

Effect of DDB1 on H_2O_2 accumulation in tomato leaves

H_2O_2, the most stable ROS, has been implicated in plant responses to *Pst* DC3000 (Parker et al. 2013). In our study, the production of H_2O_2 in AC^+, *hp1* mutant and DDB1-OE leaves was compared after *Pst* DC3000 inoculation. The DAB staining assay showed that, upon *Pst* DC3000 inoculation, less H_2O_2 accumulation was detected in the *hp1* mutant whereas more H_2O_2 accumulation was observed in the

DDB1-OE plants compared with AC^+ (Fig. 2A). ROS are known to be important secondary messengers that induce the expression of pathogenesis-related genes (Foyer & Noctor 2005). The burst of H_2O_2 can lead to cell damage or even cell death that would help to suppress the spread of pathogens and result in enhanced disease resistance (Foyer & Noctor 2005). The ROS accumulation and cell death phenotypes were compromised in the *hp1* plants and enhanced in DDB1-OE plants, suggesting that DDB1 affects the ROS-mediated responses to tomato-pathogen interactions.

DDB1 is required for the SA signaling pathway

The free SA content in AC^+ and DDB1-OE plants increased significantly ($P<0.05$) after *Pst* DC3000 inoculation but did not change in the *hp1* mutant. At 24 hpi, SA was significantly higher in DDB1-OE plants than in AC^+ ($P<0.05$; Fig. 3A). This result revealed that the SA signaling pathway was disrupted in the *hp1* mutant and promoted in DDB1-OE plants. Liu et al. (2012a) observed that exogenous SA treatment could not induce defense responses in the *hp1* mutant. As a well-known marker gene in the SA signaling pathway, *PR1a1* expression was determined at different time points after *Pst* DC3000 inoculation. This showed that *PR1a1* was significantly induced in AC^+ ($P<0.01$) and DDB1-OE ($P<0.05$) plants at 24 hpi ($P<0.05$); in the *hp1* mutant, it was also significantly higher than at 0 hpi ($P<0.01$), although this increase was only 2-fold and was notably lower than that observed in AC^+ or DDB1-OE ($P<0.05$; Fig. 4B). This confirmed that DDB1 is positively involved in the SA signaling pathway upon bacterial infection in tomato.

Salicylic acid is known to enhance the generation of ROS (Chen et al. 1993) and ROS, in turn, increases endogenous SA content (Enyedi 1999). Therefore, the suppression of H_2O_2 generation in the *hp1* mutant might be associated with SA transduction.

It has been suggested that SA caused DNA breaks after *Pst* DC3000 infection, leading to defense-related gene expression during DNA repair (Yan et al. 2013). Song & Bent (2014) also observed that *Pst* DC3000 triggered DNA breaks in *Arabidopsis*. DDB1 was identified as a damaged DNA binding protein that formed an E3–ubiquitin ligase complex with another WD40 repeat-containing protein to recognize DNA lesions and initiate DNA repair in plants and animals (Iovine et al. 2011). Therefore, the mediation of the SA signaling pathway and defense response regulation by DDB1 in tomato might be related to DNA breakage and repair upon pathogen infection.

Effect of DDB1 on the expression of pathogenesis-related genes

The expression levels of several marker genes involved in the tomato PTI pathway, including *Gras2*, *Lrr22*, *Wrky28* and *Pti5* were determined previously (Nguyen et al. 2010; Liu et al. 2012a). The expression level of *Gras2* was induced at 6 hpi in both AC^+ and DDB1-OE plants in this study, but not in the *hp1* mutant. A similar phenomenon was observed for *Wrky28* and *Lrr22* gene expression. These genes were, however, expressed notably more in DDB1-OE plants than in AC^+ plants. Unlike the other genes detected, *Pti5* was also up-regulated approximately 13-fold in the *hp1* mutant at 6 hpi compared with the base level ($P<0.01$), and was reduced at 48 hpi (Fig.

4). The expression pattern was similar to that induced by *Agrobacterium* (Liu et al. 2012a). These results showed that DDB1 played a role in the PTI signaling pathway and that PTI signaling was not totally abolished in the *hp1* mutant. This might be associated with a partial loss of function of DDB1 in the *hp1* mutant, as DDB1 is essential for normal development. In mouse and *Arabidopsis* model systems, the total deletion of DDB1 would be lethal in the early embryo stage (Cang et al. 2006; Bernhardt & Hellmann 2010).

Mitogen-activated protein kinase 3 (MPK3) is induced in DDB1-OE plants

MAPK cascades are highly conserved signaling mechanisms in eukaryotes that play essential roles in response to multiple environmental stresses (Pitzschke 2009). Several MAPKs, including MPK3, MPK6, and MPK4, are activated in *Arabidopsis* upon *Pst* DC3000 infection (Brader et al. 2007; Beckers et al. 2009). MPK3 and MPK6, which are functionally redundant and have a positive role in plant immunity responses, are two of the most important MAPK genes (Pitzschke 2009). In this study, the expression level of *MPK3* in tomato seedlings was determined through qRT-PCR. This showed that *MPK3* was rapidly reduced in DDB1-OE plants at 6 hpi ($P<0.01$); in AC^+ plants, *MPK3* was induced approximately 2.5-fold compared with the base level ($P<0.05$) at 6 hpi (Fig. 5). In the *hp1* mutant, it was not induced ($P>0.05$; Fig. 5), suggesting that the MAPK signaling pathway was compromised in the *hp1* mutant. It was reported that *MPK3* could be induced by both ROS and SA and leads to enhanced disease resistance (Miura & Tada 2014; Jalmi & Sinha 2015), whereas H_2O_2 and SA

production in the *hp1* mutant were compromised during infection. Therefore, we presumed that the tomato DDB1 protein might affect the MAPK signaling pathway through the mediation of ROS accumulation and that this was also associated with SA production. However, the interactions among DDB1, ROS, SA, and MAPK are complicated and require further investigation.

In conclusion, DDB1 played a positive role in tomato defense response. ROS accumulation, SA production, several PTI marker genes, and *MPK3* expression were compromised in a DDB1 function-deficient mutant and enhanced in the DDB1-OE plants.

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Table 1. Primers used in qRT-PCR reactions in the present study.

Gene name	Accession number	Primer sequences (5→ 3′)	Product length (bp)
PR1a1	NC015438	F: TGCTGGTGCTGTGAAGATGTG	81
		R: CAGACTTTACCTGGAGCACACG	
Pti5	NM001247058	F: ATTCGCGATTCGGCTAGACATGGT	119
		R: AGTAGTGCCTTAGCACCTCGCATT	
Lrr22	SGN-U585837	F: AAGATTGGAGGTTGCCATTGGAGC	82
		R: ATCGCGATGAATGATCGGTGGAGT	
Gras2	NM001306176	F: TAATCCAAGGGATGAGCTTCT	65
		R: CCACCAACGTGACCACCTT	
Wrky28	SGN-U586086	F: ACAGATGCAGCTACCTCATCCTCA	100
		R: GTGCTCAAAGCCTCATGGTTCTTG	
MPK3	NC015443	F: GGAGTTTTCTGATGTTTACATTGCT	343
		R: ATATTCTCGTTCTCTACGTTTGGC	
UBI3	X58253	F: TCCATCTCGTGCTCCGTCTC	160
		R: TCCTTACGAAGCCTCTGAACCT	

The primers of *PR1a1*, *Pti5*, *Gras2* and *Wrky28* were designed according to Liu et al. (2012a).

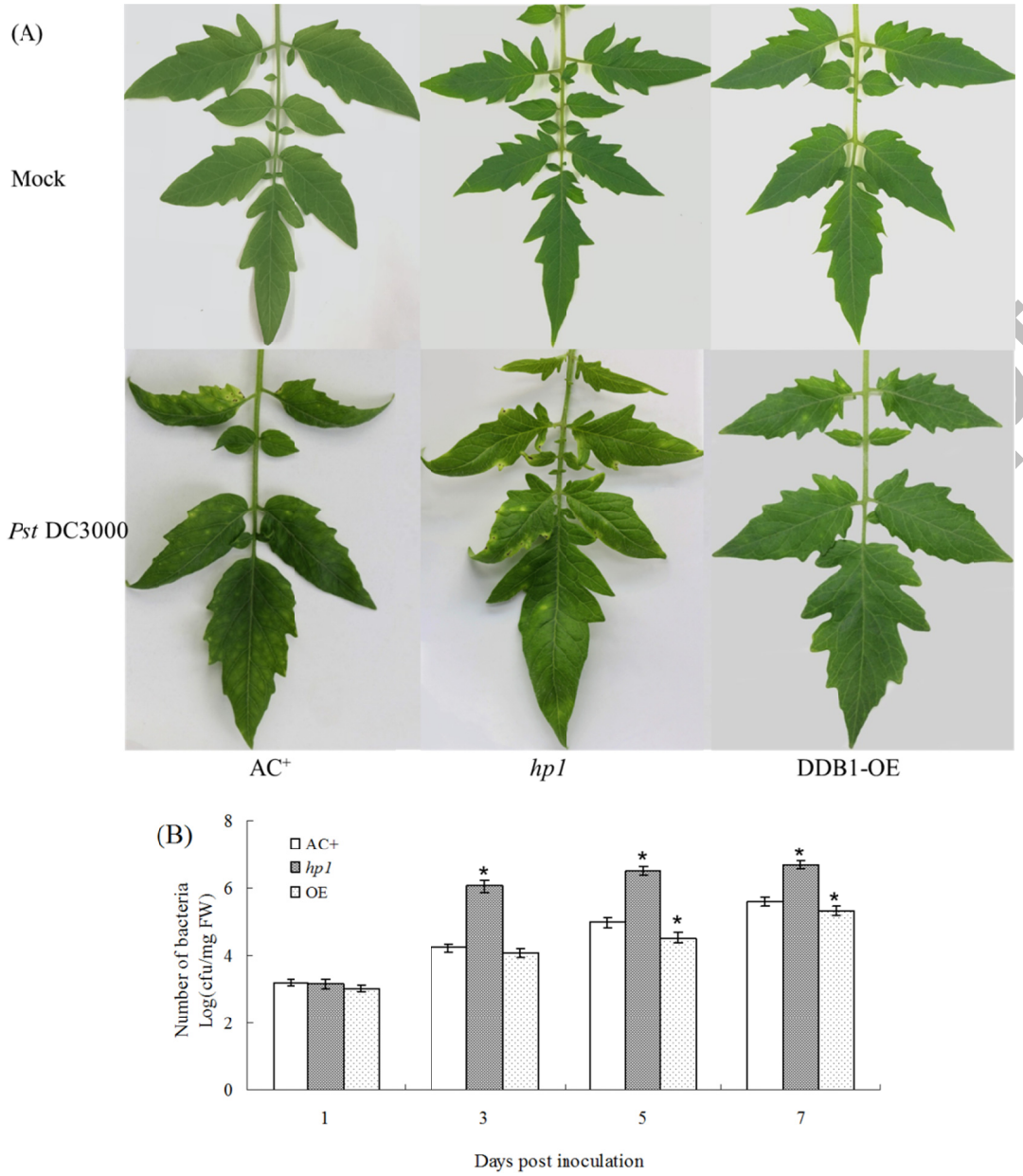

Fig. 1. Disease symptoms and bacterial growth in leaves of 'Ailsa Craig' plus (AC^+), *hp1* mutant, and DDB1 over-expression (DDB1-OE) plants upon infection with *Pst* DC3000. Photographs were taken at 7 dpi (A). Bacterial growth was determined at 0, 3, 5, and 7 dpi (B). Values followed by different letters differ significantly according to Duncan's multiple range test at $P<0.05$. FW, fresh weight; dpi, days post inoculation; * ($P<0.05$) indicated significant differences from AC^+.

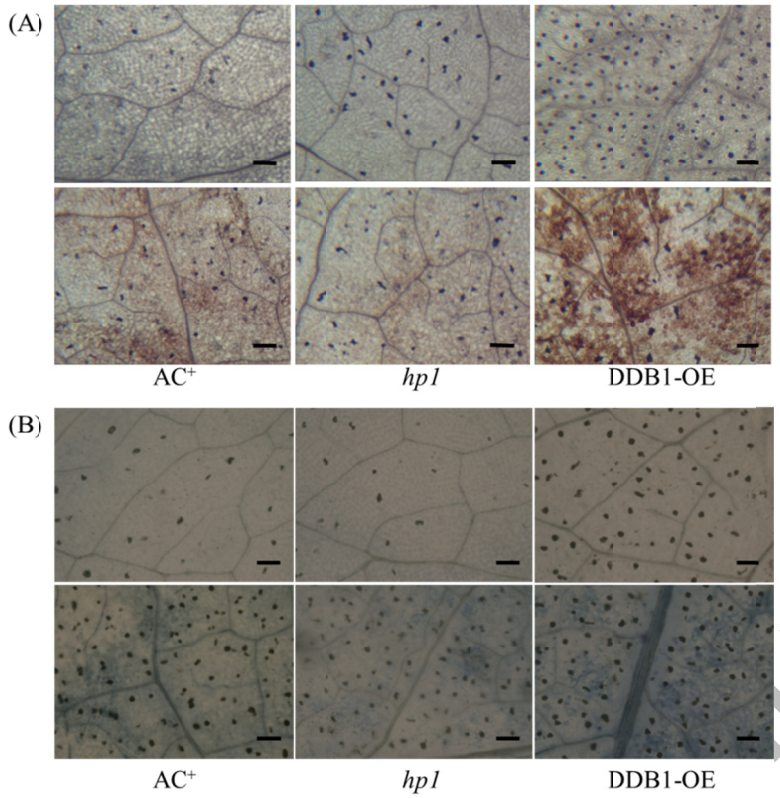

Fig. 2. Hydrogen peroxide (H_2O_2) accumulation and cell death in leaves of Ailsa Craig plus (AC^+), *hp1* mutant, and DDB1 over-expression (DDB1-OE) plants upon infection with *Pst* DC3000. The accumulation of H_2O_2 was detected by 3,3′-diaminobenzidine (DAB) staining (A) and the cell death phenotype was assayed by trypan blue staining (B) at 48 dpi. Plants were sprayed with 10 mM $MgSO_4$ (mock, top) or *Pst* DC3000 at 1×10^8 cfu mL^{-1} (bottom). dpi, day post inoculation. Bar=100 μm.

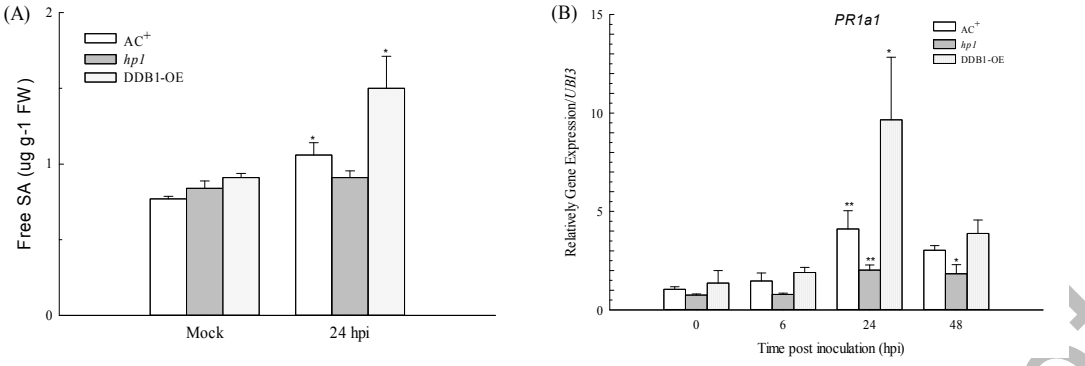

Fig. 3. Salicylic acid (SA) induction in 'Ailsa Craig' plus (AC^+), *hp1* mutant, and DDB1 over-expression (DDB1-OE) plants. Free SA production (A) was determined in leaves sprayed with 10 mM $MgSO_4$ (mock) or *Pst* DC3000 at 1×10^8 cfu mL^{-1} at 24 hpi. Expression levels of the marker gene *PR1a1* (B) were determined in leaves sprayed with *Pst* DC3000 at 1×10^8 cfu mL^{-1} at 0, 6, 24, and 48 hpi. Values followed by different letters differ significantly according to Duncan's multiple range test at $P<0.05$. FW, fresh weight; hpi, hours post inoculation; ** ($P<0.01$) or * ($P<0.05$) indicated significant differences from mock (A) or 0 hpi (B).

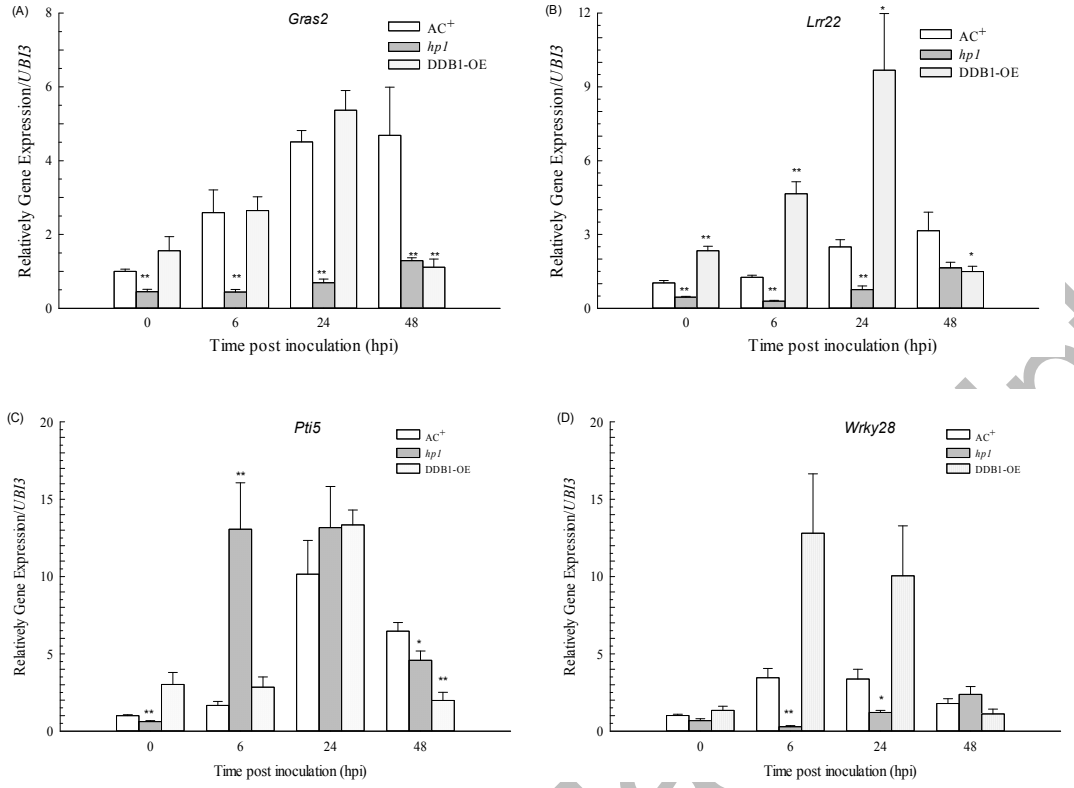

Fig. 4. Expression patterns of pattern-triggered immunity (PTI)-related genes in 'Ailsa Craig' plus (AC^+), *hp1* mutant, and DDB1 over-expression (DDB1-OE) leaves. The expression levels of *Gras2*, *Lrr22*, *Pti5*, and *Wrky28* were determined in leaves sprayed with *Pst* DC3000 at 1×10^8 cfu mL^{-1} at 0, 6, 24, and 48 hpi. Values followed by different letters differ significantly according to Duncan's multiple range test at $P<0.05$. hpi, hour post inoculation; ** ($P<0.01$) or * ($P<0.05$) indicated significant differences from AC^+.

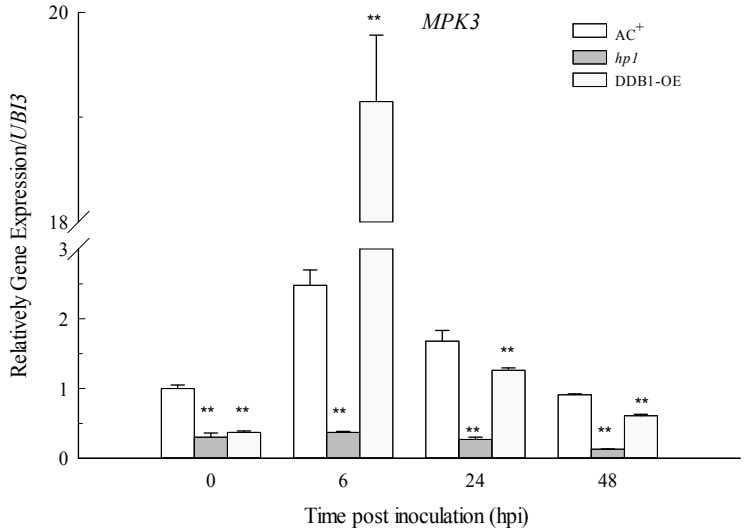

Fig. 5. Expression patterns of *Mitogen-activated protein kinase 3* (*MPK3*) in 'Ailsa Craig' plus (AC^+), *hp1* mutant, and DDB1 over-expression (DDB1-OE) plants. Gene expression levels were determined in leaves sprayed with *Pst* DC3000 at 1×10^8 cfu mL^{-1} at 0, 6, 24, and 48 hpi. Values followed by different letters are differ significantly according to Duncan's multiple range test at $P<0.05$. hpi, hours post inoculation; ****** ($P<0.01$) or ***** ($P<0.05$) indicated significant differences from AC^+.