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ORIGINAL PAPER

Overexpression of persimmon 9-lipoxygenase *DkLOX3* confers resistance to *Pseudomonas syringae* pv. tomato DC3000 and *Botrytis cinerea* in *Arabidopsis*

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Abstract Lipoxygenase (LOX) pathway initiates lipid peroxidation of cell membrane, which is crucial for plant senescence and defense pathways. Our previous study suggested that the persimmon 9-LOX gene, *DkLOX3*, promoted leaves senescence and enhanced tolerance to abiotic stresses. Here, the function of *DkLOX3* in biotic stresses was investigated in *DkLOX3*-overexpression (DkLOX3-OX) *Arabidopsis*. The results showed that inoculation with *Pseudomonas syringae* pv. Tomato (*Pst*) DC3000 in transgenic leaves exhibited more ROS accumulation, lower activity of several antioxidant enzymes and cell death phenotype in early stage, in addition, less lesions and bacterial growth were observed as compared to wild type. Quantification of jasmonic acid (JA) and salicylic acid (SA) indicated SA contents and expression of its marker gene *PR1* were increased in transgenic leaves with *Pst* DC3000 infection. Furthermore, inoculation with *Botrytis cinerea* in transgenic leaves caused more ROS accumulation, lower activity of antioxidant enzymes in early stage, however, there are no significant differences in both JA and SA contents and their marker genes expression profiles between transgenic lines and wild type. Overall, all these results indicated that *DkLOX3* plays positive roles in reducing the sensitivity to *Pst* DC3000 via regulating ROS accumulation, cell death and SA sythesis pathways, and enhancing resistance to *B. cinerea* probably by regulating ROS accumulation, but not SA or JA metabolic pathway. Our preliminary results indicated *DkLOX3* play an important role in different types of pathogens resistance.

Keywords Persimmon · 9-Lipoxygenase · Disease resistance · *Pseudomonas syringae* pv. tomato DC3000 · *Botrytis cinerea*

Introduction

Plants have evolved an array of mechanisms to protect themselves against pathogens attack and respond by activating innate immunity (Conrath et al. 2002). These responses could be activated by recognition of microbe-associated molecular patterns, damage-associated molecular patterns and effectors during infection (Boller and Felix 2009; Jones and Dangl 2006; Simone et al. 2013). Plant pathogens can be usually classified into two categories: biotrophs and necrotrophs, they have different infection cycle and plants have evolved distinct responses to feed off them (Spoel et al. 2007). It has been suggested that biotrophs caused minimal damage to the plants and obtain nutrition from living cells, while oxidative stress and programmed cell death are effective defense ways, which is triggered by recognition of R protein (Bigeard et al. 2015; Glazebrook 2005; Spoel et al. 2007). In addition, resistance mechanisms against biotrophic pathogens are associated with activation of a large set of defense genes regulated by the salicylic acid-dependent pathway (Maleck et al. 2000). In contrast, necrotrophs, depend on dead host tissues for nutrition, so they are not limited by cell death and salicylic acid-dependent defences, but a lot of researches suggested that the resistance are

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activated by jasmonic acid and ethylene signalling (Catinot et al. 2015; Dangl and Jones 2001; van Wees et al. 2003; Zander et al. 2010).

Lipoxygenases (LOXs, linoleate: oxygen oxidoreductase, EC 1.13.11.12) constitute a large gene family of non heme iron, catalyse the addition of molecular oxygen to fatty acids containing a *cis,cis*-l,4-pentadiene system to give an unsaturated fatty acid hydroperoxide (Brash 1999; Siedow 1991). In consequence, a large family of active lipid derivatives through LOX pathway were generated, collectively known as oxylipins, which are of importance in controlling abiotic stress defense and pathological processes in plants (López et al. 2008; Vellosillo et al. 2013). In plants, the primary substrates of LOX are linoleic acid and linolenic acid, and plant LOXs can be categorized as 9-LOXs or 13-LOXs depending on the position at which the oxygen is incorporated into linoleic acid or linolenic acid (Feussner and Wasternack 2002). Therefore, distinct types of oxylipins were generated from the two biosynthetic pathways.

Recently, a lot of mutants were used to elucidate the function of oxylipins from the 9-LOX pathway during pathogen infection. In *Arabidopsis* leaves, Vellosillo et al. (2007) detected that 9-HOT (9-hydroxy-10,12,15-octadecatrienoic acid) responding genes expression were up-regulated after *Pst* DC3000 inoculation, however, *noxy2* mutants, which were insensitive to 9-LOX derived oxylipins, enhanced susceptibility to *Pseudomonas* and decreased the expression level of 9-HOT- responding genes. The enhanced susceptibility of *noxy* mutants to *Pseudomonas syringae* DC3000 might by altering mitochondrial functionality (Vellosillo et al. 2013). With *noxy2* mutants and *lox1lox5* mutants lacking 9-LOX activity, Marcos et al. (2015) found that 9-LOX-derived oxylipins probably conferred resistance to *Pseudomonas syringae* by inducing brassinosteroids synthesis. In addition, the lipoxygenase action could induce structural and metabolic changes and initiate the biosynthesis of signaling molecules, which regulating the expression of plant defense genes and plant cell death (Maccarrone et al. 2001; Vellosillo et al. 2007). In pepper, a 9-LOX gene *CaLOX1*, when transiently expressed in pepper leaves, cell death and defense responses were induced, while *CaLOX1*-silenced plants enhanced sensitivity to *Xanthomonas campestris* pv. vesicatoria and *Colletotrichum coccodes*, accompanied with less cell death and reduced expression of defense-related genes (Hwang and Hwang 2010). Similarly, attenuated cell death was also observed in 9-LOX or 13-LOX silenced *Nicotiana benthamiana* after infected by the synergistic interaction of *Potato virus X* with *Potato virus Y* (García-Marcos et al. 2013). Vicente et al. (2012) found that *Arabidopsis lox1* mutants decreased the resistance to the virulent strain *Pst* DC3000, however, when pre-treated with 9-LOX-generated oxylipins, plant tissues were protected against bacterial infection by interfering with hormone homeostasis. For another, numerous 13-LOX-derived oxylipins are also regulators of plant defense to pathogen attack, including 13-hydroperoxy-10,12,15-octadecatrienoic acid (13-HPOT), 12-oxo-10,15-phytodienoic acid (12-OPDA), C6 aromatic compounds and JA, one of most understood oxylinpins (Aranega-Bou et al. 2014; He et al. 2006; Prost et al. 2005; Santino et al. 2013; Stintzi et al. 2001).

Despite the increasing experimental evidence for 9-lipoxygenase participating in defense to pathogen attack, however, the mechanisms remain not fully understood, especially for the behaviour under biotrophs and necrotrophs infection, two kinds of pathogens with different infection cycles. In our previous study, the persimmon 9-LOX gene, *DkLOX3*, was found to promote fruit ripening and leaves senescence through lipid peroxidation or accelerated ethylene production. Moreover, *DkLOX3* overexpression in transgenic *Arabidopsis* were unsusceptible to abiotic stresses via regulating reactive oxygen species accumulation and ABA-dependent or ABA-independent stress responsive genes expression. In this study, we further evaluated the effect of *DkLOX3* on biotic stress during pathogen infection using *DkLOX3*-OX transgenic *Arabidopsis*. Our results indicated that *DkLOX3* overexpression in *Arabidopsis* enhanced the tolerance to different pathogens infection through different approaches.

Materials and methods

Plant materials

Arabidopsis DkLOX3 overexpression transgenic lines were used as described previously (Hou et al. 2015). When *Arabidopsis* ecotype Columbia-0 and transgenic lines were grown in a controlled growth chamber (22 °C, 16 h light/8 h dark) for 4 week, leaf numbers 5 and 6 were detached from rosettes and placed on wet filter paper for pathogen inoculation.

Pathogen inoculation and disease assays

Pst DC3000 was grown at 28 °C in King'B liquid medium containing 50 mg/l rifampicin and 50 mg/l kanamycin, and then bacteria were collected, resuspended in 10 mM $MgCl_2$. The bacterial suspensions were adjusted to 10^7 cfu/ml (OD = 0.02). Detached leaves were sprayed with *Pst* DC3000 bacterial suspensions or 10 mM $MgCl_2$ (control), respectively. To study bacterial growth, leaf disks from six independent leaves were collected at indicated time, sterilized with 70% ethanol for 1 min, and washed three times with sterile distilled water. Then the leaf disks were ground and homogenized in 1 ml sterile distilled water and then diluted to the proper concentration and plated on King'B agar medium supplemented with 50 mg/l Rif. After 3 days, the number of bacteria colonies were counted.

Botrytis cinerea was grown at 22 °C on potato glucose agar medium for 21 days, then conidia were washed down with distilled water and the spore concentrations were adjusted to 5×10^4 conidia/ml using a hemacytometer. Detached leaves were sprayed with spore concentrations or distilled water (control), respectively.

After inoculation, detached leaves were covered with transparent plastic film to keep high humidity, incubated at 22 °C for 24 h in dark and then transferred to growth chamber. Disease symptoms were observed daily, and samples were collected at 12, 24, 48 and 72 h after inoculation for histochemical staining and H_2O_2 accumulation, moreover, another samples were frozen in liquid for further reseach.

Detection of reactive oxygen species and cell death

Accumulation of O_2^- was detected according to Kumar et al. (2014). Leaves were immersed in solution of 0.1% (w/v) nitro blue tetrazolium (NBT) in 10 mM potassium phosphate buffer (pH 7.8) and vacuum-infiltrated for 5 min followed by incubation at room temperature for 2 h in the dark. After staining with NBT, the leaves were distained with ethanol.

To monitor cell death, leaves were stained with a boiled trypan blue solution (10 ml 85% (lactic acid), 10 g phenol, 10 ml glycerol, 10 mg trypan blue, and 10 ml ddH_2O) for 5 min, and then washed with sterilized ddH_2O and bleached with 2.5 g/ml chloral hydrate (Guo et al. 2015).

After staining, samples were placed on filter paper and pictures were taken with reflected light by an Olympus BX-51 microscope (Olympus Corporation, Japan).

Quantitative assay of H_2O_2 accumulation were determined according to Zhou et al. (2006). Fresh leaves were ground and homogenized with 5 ml of 5% (w/v) trichloroacetic acid including 0.15 g activated charcoal. After being centrifuged at 12,000 g for 20 min at 4 °C, the supernatant was adjusted to pH 8.4 with 17 M ammonia solution and then filtered. A mixture of 1 ml of the filtrate and 1 ml acetic acid buffer were incubated at 30 °C for 10 min, then the absorbance at 505 nm were measured using a UV-1800 spectrophotometry (Shimadzu, Kyoto, Japan). The filtrate digested with 8 mg catalase at room temperatures for 10 min was served as blank. The colorimetric reagent was 50 ml of 100 mM acetic acid buffer (pH 5.6), including 10 mg phenol, 10 mg 4-aminoantipyrine and 5 mg peroxidase (150 U mg^{-1}).

Activities measurement of antioxidant enzymes and lipoxygenase

The peroxidase (POD) activity was measured according to Fang and Kao (2000), with slight modifications. Frozen samples were ground and homogenized in 10.0 ml of 0.1 M ice-cold sodium phosphate extraction buffer (pH 7.0), including 1 mmol PEG, 1% (v/v) Triton X-100 and 4% (w/v) polyvinylpyrrolidone (PVP). After being centrifuged, the supernatant was used for POD enzyme activity determinations following the oxidation of 25 mmol guaiacol by measuring the absorbance at 470 nm using UV-1800 spectrophotometry. One unit of POD activity was defined as a change in the absorbance of 0.01 min^{-1} using guaiacol as a substrate.

Crude protein extracts for measuring catalase (CAT) and superoxide dismutase (SOD) were activities were isolated under modified protocols described by Zhou et al. (2005). Frozen samples were ground and homogenized in 10.0 ml of 0.1 M ice-cold sodium phosphate extraction buffer (pH 7.0) containing 5 mmol DTT and 5% (w/v) PVP, followed by centrifugation at 12,000 g for 15 min at 4 °C. CAT activity were measured following the catalysis of 6.67 mmol H_2O_2 by testing the absorbance at 240 nm. One unit of CAT activity was defined as a change in the absorbance of 0.01 min^{-1}. For SOD activity, the absorbances of reaction mixtures comprising 1.7 ml of 0.1 M sodium phosphate buffer (pH 7.0), 0.1 ml of the supernatant and 0.3 ml of each reaction component, including 13 mmol L-methionine, 0.1 mol EDTA disodium, 75 µmol NBT and 2 µmol riboflavin were detected at 560 nm.

The LOX activity was measured according to Hou et al. (2015). One unit of LOX activity was defined as a change in the absorbance of 0.01 min^{-1}.

Measurement of SA and JA

The methods for extraction and purification of JA and SA were conducted according to Yang et al. (2001). Quantification of hormones was carried out according to the protocols described by ELISA produced at the Phytohormones Research Institute (made by China Agricultural University). Standard buffer was diluted to different grad and standard curve was draw according to the absorbance. ELISA was performed on a 96-well microtiter plate, and samples extraction solution were added to each well, enveloped with microplate sealers and incubated for 30 min at 37 °C, then washed for five times by cleaning solution. After that, each well was added into 50 µl elisa regent, sealed and incubated for 30 min followed by washing with cleaning solution for five times. Then, color-developing agent A and B were added into the well successively, blended gently and reacted in dark for 10 min. The reaction progress was stopped by adding of stop buffer with the colour of reaction reagent turning to yellow from blue. The absorbance of reaction reagent of each well was measured at 450 nm, and the concentration of JA and SA was calculated following the standard curve.

Expression analysis by qPCR

Total RNA was extracted from *Arabidopsis* leaves were extracted using a TransZol Up Plus RNA Kit (Transgen Biotech, Beijing, China). The first-strand cDNA was synthesized from 1 μg of RNA using the PrimeScriptTM RT Reagent Kit with the gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's protocol. The synthesized cDNA was diluted 20-fold for the following qPCR analysis.

qPCR was performed in 20 μl reactions containing 2 μl of diluted cDNA samples and 0.8 μl of gene-specific primers (10 μM), 6.4 μl of ddH2O and 10 μl of SYBR Premix Ex TaqTMII, under the following conditions: an initial hot start at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s and 72 °C for 20 s, and then completed with a melting curve analysis program. *AtACTIN2* were used as reference gene.

The primers that were used forqPCR are shown in Supplemental Table 1. The primers were designed using Primer Premier 5 software.

Statistical analysis

All of the experiments were repeated three times, and each replicate included at least three technological replicates. The results are represented as the mean ± standard errors. Significant differences among the means were assessed by analysis of one way ANOVA with the least significant difference (LSD) at $P < 0.05$ using SPSS 16.0 software.

Results

Overexpression of *DkLOX3* in *Arabidopsis* enhanced resistance to *Pst* DC3000

Bacteria *Pst* DC3000 was used first to examine the effects of *DkLOX3* overexpression on the pathogen resistance. As shown in Fig. 1a, detached leaves from wild types exerted apparent disease lesions at 3 days after inoculation, however, significantly limited disease symptoms were observed in transgenic leaves. NBT, trypan blue staining and H_2O_2 quantification showed transgenic leaves exhibited ROS burst and more cell death in the first 24 h, which showed a typical hypersensitive reaction (HR). However, in the following time, ROS accumulation of transgenic leaves was decelerated, while that in wild type were accumulated rapidly and more than transgenic lines (Fig. 1b–d). At 48 and 72 h, bacterial population in the inoculated leaves from wild type and transgenic lines were tested. The results showed growth of *Pst* DC3000 in wild type were more severely, approximately 30, 36 and 38 folds of transgenic lines (Fig. 1e).

Antioxidant enzymes and LOX activities in *DkLOX3*-OX transgenic *Arabidopsis* against *Pst* DC3000

After inoculated with *Pst* DC3000, transgenic leaves exhibited more ROS accumulation and cell death, therefore activities of antioxidant enzymes, including POD, SOD and CAT were further analyzed. Activities of POD, SOD and CAT in the transgenic lines and wild type were both increased after inoculation, reached a peak and then decreased (Fig. 2). Compare with wild type, POD and CAT enzyme activities in transgenic lines were improved slowly and maintain a lower level in the first 12 and 24 h after inoculation, however, at 48 h, the activities were increased faster and decreased slowly at 72 h after inoculation (Fig. 2a, c). Activities of SOD were increased at the early stage after inoculation, nevertheless, the activities decreased sharply after 24 h, while that in transgenic lines was declined steady after inoculation (Fig. 2b).

Lipoxygenase activities in wild type and transgenic lines revealed an uniform trend, activities reached a peak at 12 h after inoculation and then decreased. Across all the time accord, the activities of lipoxygenase in transgenic lines were significantly higher than wild type (Fig. 2d).

Enhanced SA-induced defense response to *Pst* DC3000 in *DkLOX3*-OX transgenic *Arabidopsis*

To elucidate the possible mechanism involved in the enhanced resistance in *DkLOX3*-OX *Arabidopsis* plants, we analyzed and compared the accumulation of JA, SA and the expression profiles of their marker genes. JA contents in transgenic lines were a little lower than wild type, but there is no significant difference (Fig. 3a). SA content of transgenic lines and wild type were increased and reached a peak at 24 h after inoculation, nevertheless, SA contents in transgenic lines were always higher than wild type (Fig. 3b).

Furthermore, qPCR analysis showed that transcript abundance of JA marker gene *PDF1.2* in transgenic lines was nearly identical to wild type (Fig. 3c). In addition, expression levels of SA marker genes *PR1* were significantly higher in the transgenic leaves than that of wild-type (Fig. 3d).

Overexpression of *DkLOX3* in *Arabidopsis* enhanced resistance to *B. cinerea*

To further confirm the function of *DkLOX3* in disease resistance, we examined whether overexpression of *DkLOX3* could confer an increased resistance to *B. cinerea*, a necrotrophic fungal pathogen. At 72 h after

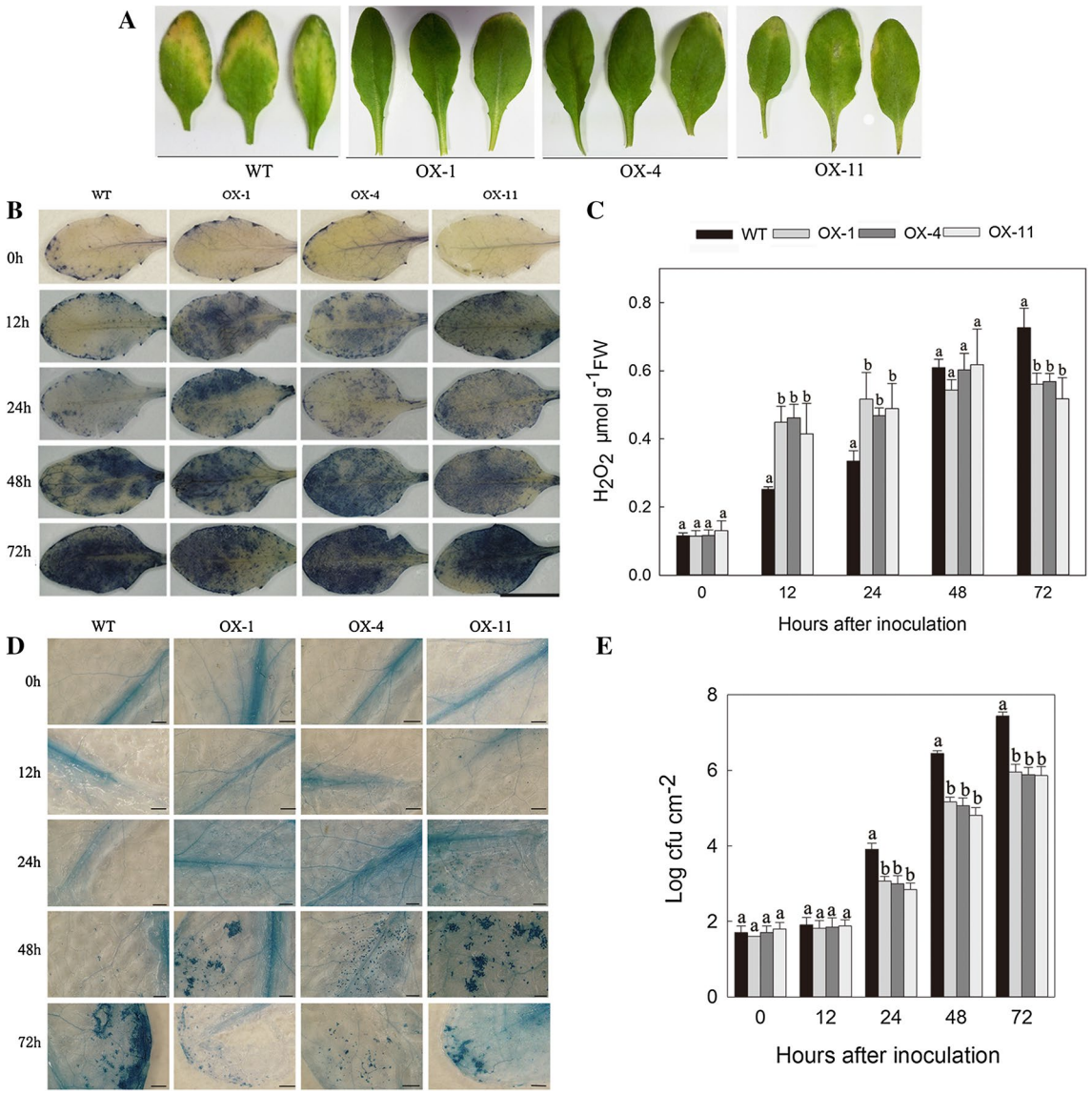

Fig. 1 Ehanced resistance of *DkLOX3*-OX *Arabidopsis* transgenic lines (OX-1, OX-4 and OX-11) to *Pst* DC3000 infection. **a** Disease symptoms on leaves infected with *Pst* DC3000. Leaf numbers 5 and 6 were exicised from 4-week-old rosettes after sowing. **b** Levels of O_2^- accumulation detected with NBT staining in leaves at 0, 12, 24, 48 and 72 h after inoculation, respectively. Bars = 1 cm. **c** Trypan blue staining of cell death in leaves after inoculation, respectively. Bars = 2 mm. **d** Quantification of H_2O_2 in leaves at 0, 12, 24, 48 and 72 h after inoculation, respectively. **e** Bacterial growth in leaves after inoculation, respectively. Data in (**d**) and (**e**) are presented as mean ± standard error from three replicates with three biological repeats and different letters above the columns indicate significant differences (LSD, $P < 0.05$) between wild type and transgenic lines

inoculation, apparent disease symptom was observed in leaves of wild type, however, disease spots were faintly visible in transgenic leaves (Fig. 4a). NBT staining showed that O_2^- accumulation was higher in transgenic leaves than wild type at 12 and 24 h after infection, afterwards, O_2^- accumulation in transgenic lines was retarded and aggregated at lesion area, however, O_2^- in wild type was quickly accumulated in all areas (Fig. 4b). Quantification of H_2O_2 presented the similar trend with O_2^- accumulation (Fig. 4c).

Antioxidant enzymes and LOX activities in *DkLOX3*-OX transgenic *Arabidopsis* against *B. cinerea*

POD, SOD and CAT activities in wild type were decreased since 12 h after infection, while that of transgenic lines were increased firstly and peaked at 48, 24 and 24 h, respectively (Fig. 5a–c). At 12 h after infection, activities of these three enzymes in wild type were higher than transgenic lines, while in the following time, enzyme activities of transgenic

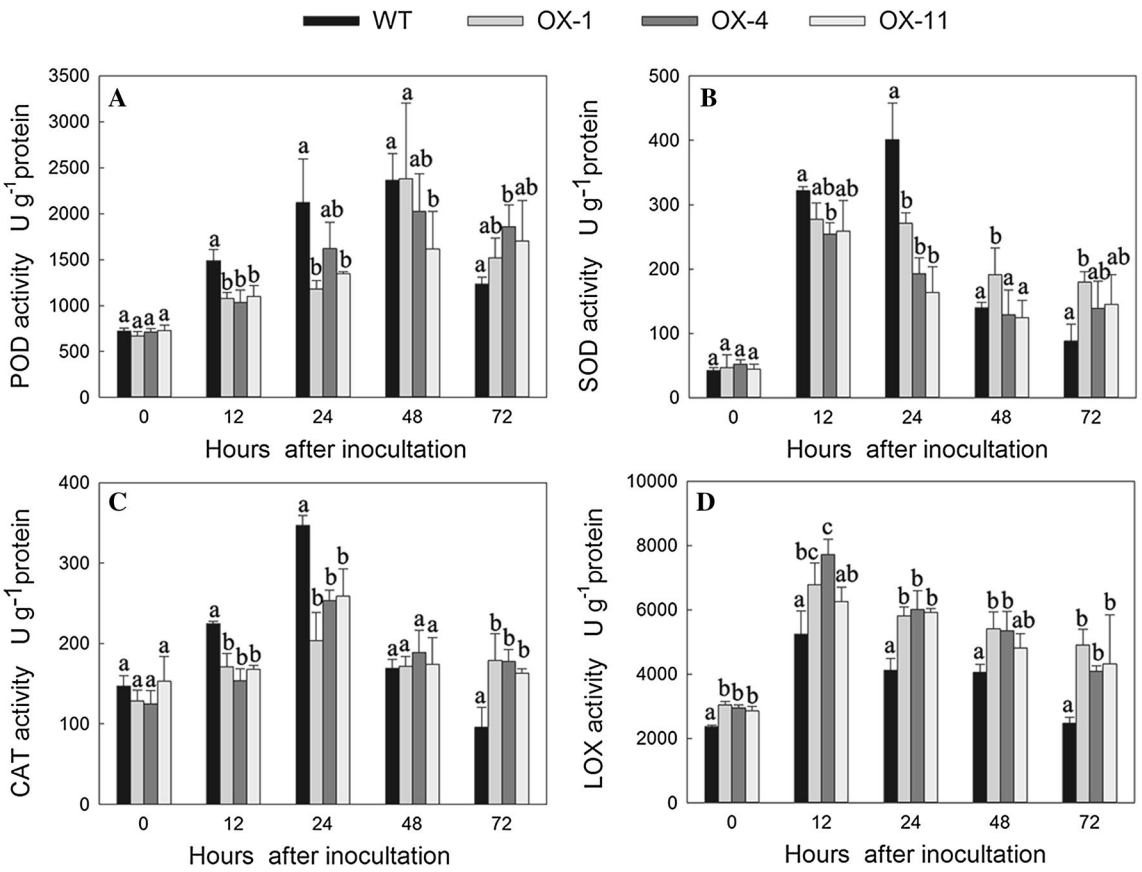

Fig. 2 Activities of *POD* peroxidases (**a**), *CAT* catalase (**b**), *SOD* superoxide dismutase (**c**) and *LOX* lipoxygenase (**d**) in detached leaves infected with *Pst* DC3000. Leaf numbers 5 and 6 were excised from 4-week-old rosettes from *WT* wild type and transgenic lines (OX-1, OX-4 and OX-11). Data are presented as mean ± standard error from three replicates with three biological repeats and different letters above the columns indicate significant differences (LSD, $P < 0.05$) between wild type and transgenic lines

leaves were increased gradually and exceeded that of wild type and maintain at a higher level (Fig. 5a–c). Lipoxygenase activities in both wild type and transgenic lines were induced at 12 h after infection and then declined, however, lipoxygenase activities in leaves from transgenic lines were always higher than wild type due to the constitutive expression of *DkLOX3* (Fig. 5d).

Discussion

Numerous studies have shown that leaf senescence has a substantial crosstalk with plant defense signaling pathways (Buchanan-Wollaston et al. 2005; He et al. 2002; Spoel et al. 2007; Ülker et al. 2007; Jing et al. 2009). Our previous study also showed that the persimmon *DkLOX3* plays a positive role in both promoting leaf senescence and decreasing susceptibility to osmotic, salinity and drought stresses in *DkLOX3*-OX *Arabidopsis* (Hou et al. 2015). In this work, investigation of the role of *DkLOX3* in response to biotic stresses revealed that *DkLOX3* overexpression also enhanced the resistance to *Pst* DC3000 and *Botrytis cinerea*, which represent a hemibiotrophic bacterial pathogen and a necrotrophic fungal pathogen, respectively. Furthermore, the possible defense pathways were studied, and the results indicated that *DkLOX3*-OX *Arabidopsis* plants raised the resistance to *Pst* DC3000 via regulating ROS accumulation, HR cell death and SA biosynthetic pathways, and decreased the susceptibility to *Botrytis cinerea* by regulating ROS accumulation possibly via the defense pathways independent of JA.

Following inoculation with the hemibiotrophic bacterial pathogen *Pst* DC3000, the transgenic leaves were rapidly accumulated more ROS and dead cells (Fig. 1b–d), which was established as HR. This hypothesis is supported by previous studies. Hwang and Hwang (2010) found that *CaLOX*1-transient expression in pepper plants could accumulate ROS and phenolic compounds, which were associated with cell death. Moreover, in plant-pathogen interactions, Jalloul et al. (2002) and Montillet et al. (2002) found HR cell death was occurred and had a correlation with LOX-mediated lipid peroxidation and oxylipins metabolism. As a result, HR cell death markedly reduced the bacterial growth

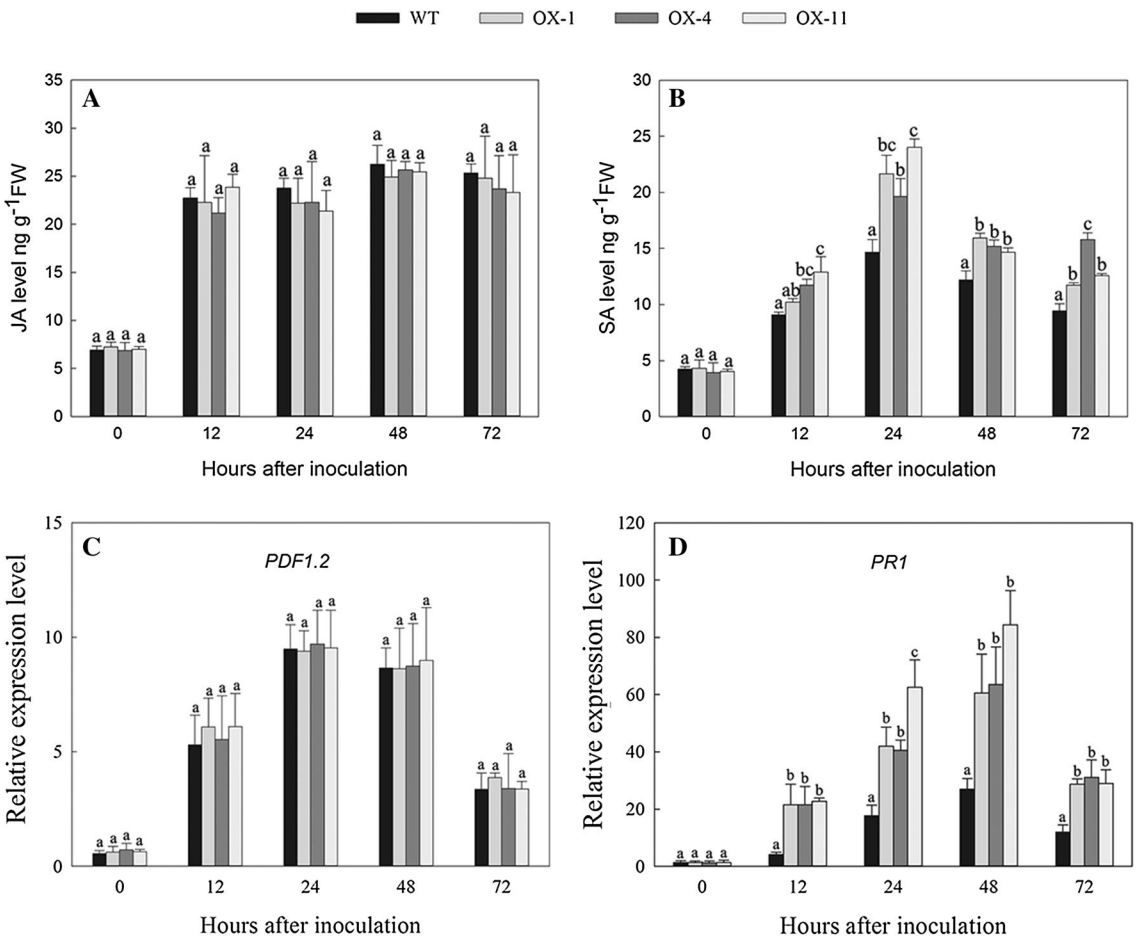

Fig. 3 Quantification of Jasmonic acid (**a**), salicylic acid (**b**) and expression level of defense-related genes *PDF1.2* (**c**) and *PR1* (**d**) in detached leaves at 0, 12, 24, 48 and 72 h after *Pst* DC3000 inoculation, respectively. Leaf numbers 5 and 6 were exicised from 4-week-old rosettes from *WT* wild type and transgenic lines (OX-1, OX-4 and OX-11). Data are presented as mean ± standard error from three replicates with three biological repeats and different letters above the columns indicate significant differences (LSD, $P < 0.05$) between wild type and transgenic lines

and the lesions in the transgenic leaves, as compared to those observed in WT plants (Fig. 1a, e). In addition, several researches declared oxidative burst was a common response to virtually every biotic stress (Niu et al. 2016; Overmyer et al. 2003; Vranová et al. 2002; Wang et al. 2013), and these agree with our results. After inoculation, more O_2^- and H_2O_2 were accumulated in transgenic leaves, which might be attributed for the lower activities of antioxidant enzymes including POD, SOD and CAT at 12 and 24 h after inoculation (Fig. 2a–c). It indicated that *DkLOX3* overexpression-induced ROS might be crucial for HR cell death, and this is agreed with the results of previous studies. Hwang and Hwang (2010) found that *CaLOX1*-silenced pepper plants decreased ROS accumulation and *CaLOX1* overexpressing *Arabidopsis* increased ROS accumulation accompanying with weakened and enhanced resistance to pathogens, respectively. Moreover, LOX-derived oxylipins are also considered as important regulators in defense. In potato hyper-sensitively reacting cells, lipid peroxidation mediated by 9-LOXs was increased (Göbel et al. 2003). In addition, Marcos et al. (2015) found that 9-LOX mutants *lox1lox5* and oxylipins nonresponding mutant *noxy2-2* enhanced the susceptibility to *Golovinomyces cichoracearum*, an obligate biotrophic fungus.

Apart from the roles of ROS and LOX-derived oxylinpins in defense to biotrophs, a lot of studies have provided strong evidences for that SA as signal molecule inducing resistance against biotrophic pathogens via activation of a large set of pathogenesis-related (*PR*) genes (Hu et al. 2012; Luna and Ton 2012). Our results also showed that *Pst* DC3000 infection significantly increased SA synthesis and expression level of SA marker genes of transgenic lines, compared to those of wild type, while JA contents were not influenced (Fig. 3). This is consentient with previous studies. Pepper *CaLOX1*-silenced plants decreased SA accumulation and exhibited more susceptiblity to

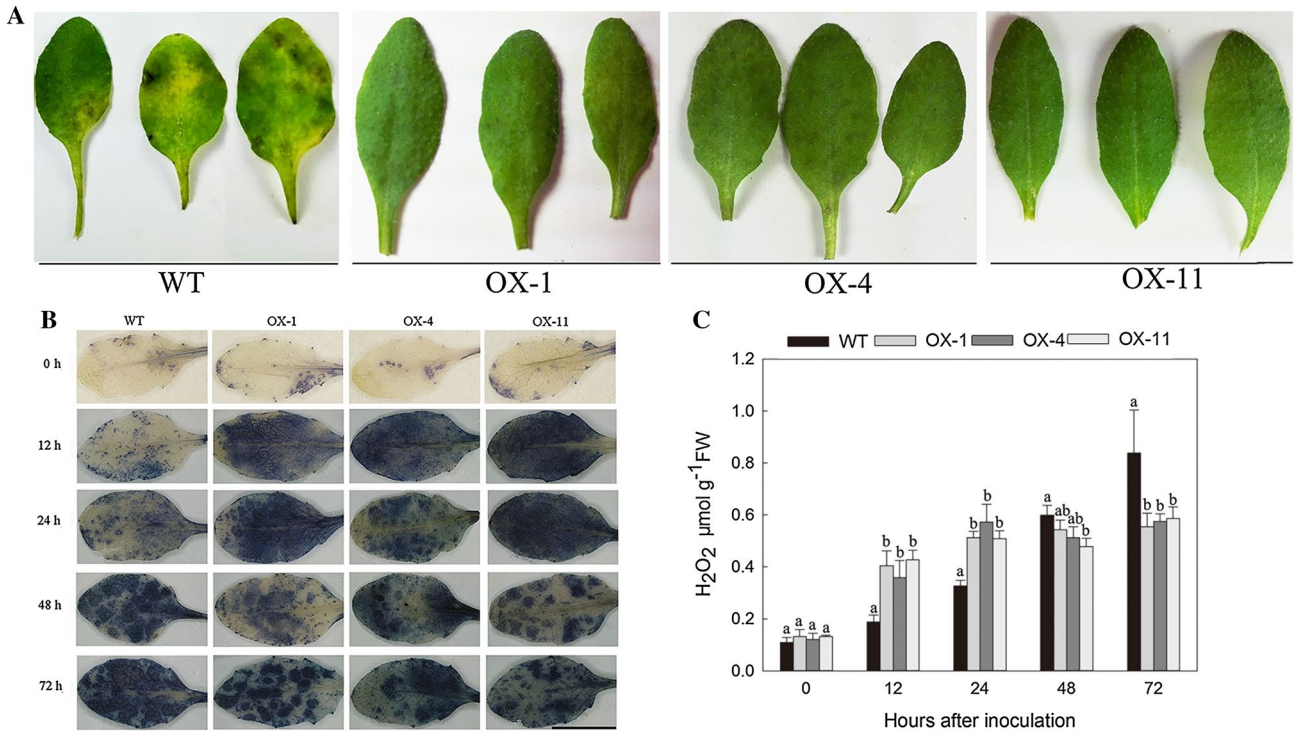

Fig. 4 Enhanced resistance of *DkLOX3*-OX *Arabidopsis* transgenic lines (OX-1, OX-4 and OX-11) to *Botrytis cinerea* infection. **a** Disease symptoms on leaves infected with *Botrytis cinerea*. Leaf numbers 5 and 6 were exicised from 4-week-old rosettes after sowing. **b** Levels of O_2^- accumulation detected with NBT staining in leaves at 0, 12, 24, 48 and 72 h after inoculation, respectively. Bars = 1 cm. **c** Quantification of H_2O_2 in leaves at 0, 12, 24, 48 and 72 h after inoculation, respectively. Data in (**c**) are presented as mean ± standard error from three replicates with three biological repeats and different letters above the columns indicate significant differences (LSD, $P < 0.05$) between wild type and transgenic lines

Xcv and *C. coccodes*, while *CaLOX1*-OX *Arabidopsis* increased SA content and conferred to enhanced resistance to *Pst* DC3000, *Hyaloperonospora arabidopsidis* and *Alternaria brassicicola* (Hwang and Hwang 2010). Moroever, enhanced susceptibility to the virulent strain *Pst* DC3000 was observed in *Arabidopsis lox1* mutant, while oxylinpin 9-ketooctadecatrienoic acid (9-KOT) produced by 9-LOX could dimish the defect and limit bacterial growth. Meanwhile, with 9-KOT pre-treated *Pst* DC3000-infected tissues, 22 and 55% of up-regulated genes were responding to SA and oxidative stress, respectively (Vicente et al. 2012). Overall, it is suggested that *DkLOX3* probably plays a positive role in enhancing to the resistance to *Pst* DC3000 via regulating ROS accumulation, HR cell death and SA synthesis. In addition, recent studies suggest that ethylene signalling was activated in inoculated *N. benthamiana* leaves by *Phytophthora parasitica*, a hemibiotrophic pathogen (Shen et al. 2016; Sun et al. 2016). In our previous research, *DkLOX3* played positive roles in promoting tomato fruit ripening through accelerated ethylene production (Hou et al. 2015), but in this regard, it is not clear that whether *DkLOX3* is involved in *Pst* DC3000 resistance via ethylene signalling.

As it is mentioned, plant usually defense biotrophic pathogens via programmed cell death to limit pathogen growth, and this is benefit for necrotrophs, which acquired for nutrition from dead host tissue (Catinot et al. 2015). Guo et al. (2016) found that constitutive expression of grape *AP13* in *Arabidopsis* enhanced the resistance to biotrophic pathogen *E. necator* but increased susceptibility to necrotrophic pathogen *Botrytis cinerea*, and this was supported by Mang et al. (2009) and Mitsiades et al. (2006). Moreover, Vicente et al. (2012) found that in 9-KOT induced protect systems to *Pst* DC3000, 22 and 55% of total up-regulated genes were respond to SA and oxidative stress, while 35 and 25% of down-regulated genes were respond to ABA and MeJA. Enhanced disease resistance was excerted in *Arabidopsis lox1* and *lox5* mutants, correlating with robust induction of SA accumulation and reduction of JA signaling during *Fusarium graminearum* infection, which was performed as a transient biotrophic phase during early stages and followed switching to necrotrophy (Nalam et al. 2015). These results indicated the antagonistic interaction in plant defense to biotrophs and necrotrophs according to the tradeoffs of JA and SA (Spoel et al. 2007). Nevertheless, our results showed that the resistance to necrotrophic pathogen *Botrytis cinerea*

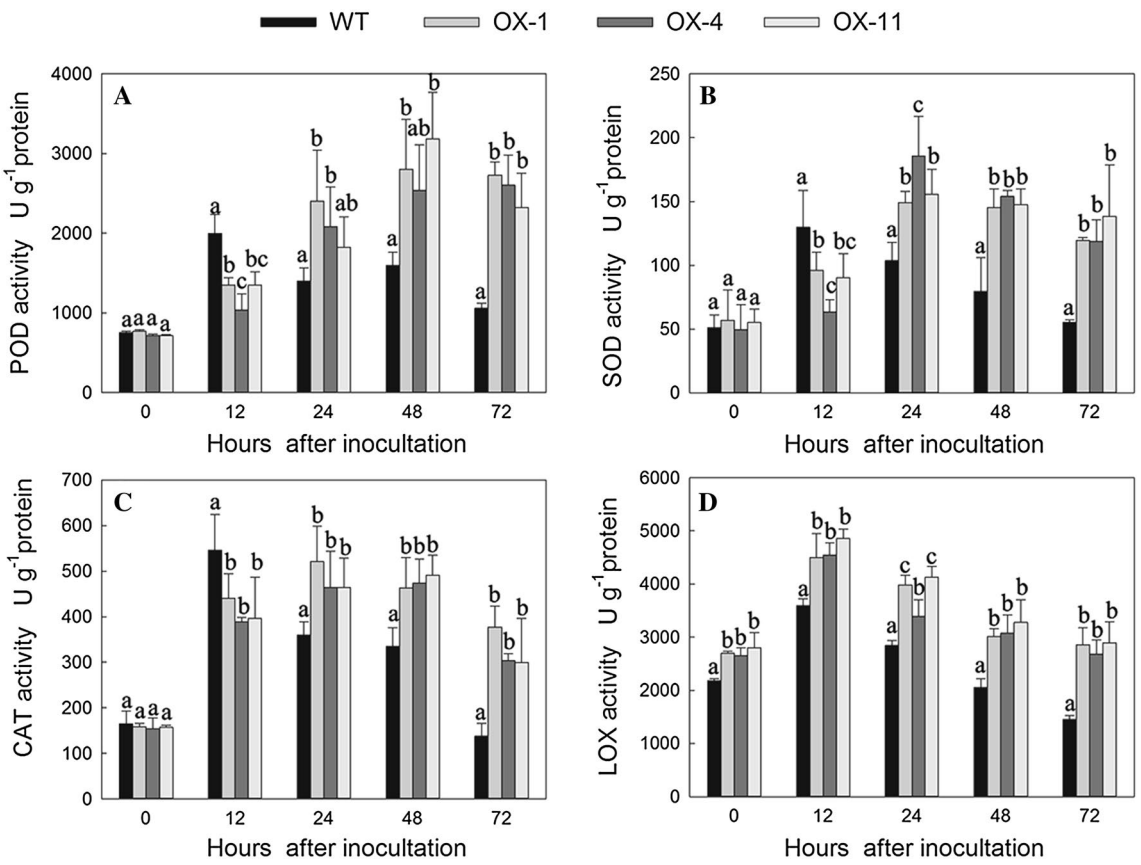

Fig. 5 Activities of *POD* peroxidases (**a**), *CAT* catalase (**b**), *SOD* superoxide dismutase (**c**) and *LOX* lipoxygenase (**d**) in detached leaves infected with *Botrytis cinerea*. Leaf numbers 5 and 6 were exicised from 4-week-old rosettes from *WT* wild type and transgenic lines (OX-1, OX-4 and OX-11). Data are presented as mean ± standard error from three replicates with three biological repeats and different letters above the columns indicate significant differences (LSD, $P < 0.05$) between wild type and transgenic lines

was also enhanced, and this might be attributed to the broad involvement of *DkLOX3* in regulation of disease resistance against different pathogens. Furthermore, this general resistance to both bacteria and fungus have also been found in previous studies. Tomato *Sl3-MMP* and *Arabidopsis ERF15* have both shown a positive regulator of multiple layers of the immune responses with different mechanisms (Li et al. 2015; Zhang et al. 2015).

There have been a series of studies proposed the necrotrophic pathogens reproduction were sufficiently inhibited by JA (Glazebrook 2005; Kliebenstein and Rowe 2008; Pieterse et al. 2012; Spoel and Dong 2008). However, our results showed that the enhanced resistance to *Botrytis cinerea* was accompanied with early ROS burst (Fig. 5), while no obvious variance was detected in JA or SA contents and their marker genes expression profiles, when compared to wild type (Supplemental Fig. 1). In addition to JA, ethylene (ET) has been also known as associating with defence against necrotrophic pathogens (Bari and Jones 2009). Ethylene responsive factor *ERF96* and *ERF1* were demonstrated to act as possible player in the resistance of *Arabidopsis* plants to necrotrophic pathogens (Berrocal-Lobo et al. 2002; Catinot et al. 2015). Moerover, *ERF1*-OX transgenic wheat exhibited significantly enhanced resistance to necrotrophic pathogen *Rhizoctonia cerealis* (Zhu et al. 2014). We also previously demonstrated that *DkLOX3* overexpression could accelerate ethylene biosynthesis (Hou et al. 2015). Combined those researches with our previous studies, we proposed the hypothesis that *DkLOX3* might confer the resistance to necrotrophic pathogen by influencing the ethylene signal pathways, and this should be verified with further research.

In summary, characterization of persimmon *DkLOX3* in disease resistance was studied in transgenic *Arabidopsis*. Our results showed that *DkLOX3* overexpression might contribute resistance to general bacteria and fungus. In detail, *DkLOX3* overexpressed transgenic lines enhanced tolerance to *Pst* DC3000 through regulating ROS accumulation, HR cell death and SA transduction pathway. Futhermore, *DkLOX3* functions as a positive regulator of defense response against *Botrytis cinerea* via regulating ROS accumulation. However, the biological functions of *DkLOX3*

in reponse to *Botrytis cinerea* were also deficient, and this should be investigated with further research.

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