Exogenous Calcium Improved Resistance to *Botryosphaeria dothidea* by Increasing Autophagy Activity and Salicylic Acid Level in Pear

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Pear ring rot, caused by *Botryosphaeria dothidea*, is one of the most serious diseases in pear. Calcium (Ca^{2+}) was reported to play a key role in the plant defense response. Here, we found that exogenous calcium could enhance resistance to *B. dothidea* in pear leaves. Less H_2O_2 and O_2^- but more activated reactive oxygen species scavenge enzymes accumulated in calcium-treated leaves than in H_2O -treated leaves. Moreover, the increased level of more ascorbic acid-glutathione was maintained by Ca^{2+} treatment under pathogen infection. The expression of core autophagy-related genes and autophagosome formations were enhanced in Ca^{2+} -treated leaves. Silencing of *PbrATG5* in *Pyrus betulaefolia* conferred sensitivity to inoculation, which was only slightly recovered by Ca^{2+} treatment. Moreover, the salicylic acid (SA) level and SA-related gene expression were induced more strongly by *B. dothidea* in Ca^{2+} -treated leaves than in H_2O -treated leaves. Taken together, these results demonstrated that exogenous Ca^{2+} enhanced resistance to *B. dothidea* by increasing autophagic activity and SA accumulation. Our findings reveal a new mechanism of Ca^{2+} in increasing the tolerance of pear to *B. dothidea* infection.

Keywords: autophagy, *Botryosphaeria dothidea*, calcium, pear, salicylic acid

Calcium plays a key role in pear resistance to *Botryosphaeria dothidea* by increasing autophagy activity and salicylic acid level. Like all living things, the environmental challenges faced by plants can be biological, such as interactions with pathogens, or nonbiological, such as drought, extreme temperatures, and

mechanical damage. To adapt to adverse growth conditions, plants have evolved a series of strategies to activate appropriate physiological responses. As a second messenger, calcium (Ca^{2+}) is widely examined for its protective role in the response to most abiotic stresses, including drought, cold, heat, heavy metals, and oxidative stresses, and particularly in the immune response to pathogens (Aldon et al. 2018; Dai et al. 2012; Khan et al. 2010; Liu et al. 2014). The intracellular calcium concentration can be stimulated by pathogen infection. To be involved in plant defense system, the stimulated Ca^{2+} messages need to be decoded and relayed to activate the appropriate cellular response, which is carried out by a Ca^{2+} binding protein called a Ca^{2+} sensor (Steinhorst and Kudla 2014). Moreover, Ca^{2+} sensors are mandatory; for example, *Arabidopsis* is estimated to have more than 250 Ca^{2+} sensor members (Day et al. 2002). The plant Ca^{2+} sensors were classified into four major groups: the calneurin B-like group (Luan 2009), the Ca^{2+} -dependent protein kinase group (Cheng et al. 2002), the calmodulin group (Yang and Poovaiah 2003), and its closely related group, the calmodulin-like protein (CML) family (Bender and Snedden 2013). To date, the role of most Ca^{2+} sensors is largely unknown, but recent studies have indicated some roles of Ca^{2+} sensors in plant development, abiotic stress responses, and immunity (Rao et al. 2014; Yuan et al. 2017; Zeng et al. 2015). For instance, *Arabidopsis* CALMODULIN-BINDING PROTEIN60 (CBP60) is involved in the calmodulin-dependent activation of salicylic acid (SA) signaling in response to pathogen recognition (Wang et al. 2009). Moreover, CML46 and CML47 are associated with CBP60a and were reported to negatively control SA accumulation and contribute to resistance to the pathogen *Pseudomonas syringae* (Lu et al. 2018). Bundó and Coca (2016) found that the overexpression of *OsCPK4* improved resistance to blast disease in rice. Liu et al. (2017) reported that CPK5 links with TIR-NBS2 were needed for *Arabidopsis* immunity. Although recent studies have investigated the in vivo role of calcium when plants are exposed to abiotic stress or in transgenic overexpression plants, the traditional and classical methods is utilizing exogenous calcium. Application of exogenous calcium improved the inhibition of growth and development and maintained the integrity of cell function and structure under abiotic stress. For example, CaCl_2 treatment restricted the development of brown spots on pear by improving the structural integrity of the cell membrane (Kou et al. 2015). Exogenous calcium treatment increases biomass and delays the senescence of broccoli microgreens (Kou et al. 2014). In mammal, an increase in calcium induced autophagy under several physiologic and pathologic conditions (Filippi-Chiela et al. 2016; Høyer-Hansen et al. 2007; Kondratskyi et al. 2018).

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However, the molecular mechanism through which calcium promotes resistance to pathogen infection in plants is largely unknown.

Autophagy, a conserved cellular process in eukaryotes, is a universal vesicular mechanism that facilitates the degradation

Fig. 1. The diameter of disease spots of leaves treated with different concentrations of Ca²⁺. Diameter of disease spots was detected at 10 days after inoculation. Data are means of three replicates with standard error. Values not followed by the same letter indicate significant differences between treatments, according to one-way analysis of variance Tukey's multiple range tests ($P < 0.05$).

of unwanted cell constituents in eukaryotic cells and plays an important role in recycling nutrients and cytoplasmic components as well as in conferring tolerance to biotic and abiotic stresses (Farré and Subramani 2016; Han et al. 2015; He and Klionsky 2009; Yang and Bassham 2015). To date, more than 35 autophagy-related (*ATG*) genes have been identified in yeast, and most of them have close homologs in plants (Liu and Bassham 2012; Shibutani and Yoshimori 2014). With the characterization of plant *ATG* genes, the role of autophagy has been widely revealed, including plant abiotic and biotic stresses, such as seed germination, development, senescence, drought, salt, and plant-pathogen interactions (Chung et al. 2009; Hofius et al. 2017; Qi et al. 2017; Yang et al. 2019). There is convincing evidence that autophagy is required for plant resistance to necrotrophic pathogens. The *Arabidopsis* mutant *atg18a* was more hyposensitive to the necrotrophic fungal pathogen *Botrytis cinerea* (Lai et al. 2011). The *Arabidopsis* autophagy-deficient mutants *atg5*, *atg10*, and *atg18a* have spreading necrosis areas when infected by *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Lenz et al. 2011), while *Arabidopsis bag6* (*Bcl-2-associated athanogene 6*) mutants are defective in autophagosome formation and hypersusceptible to *B. cinerea* (Li et al. 2016). These results indicated that autophagy plays a crucial role in plant resistance to necrotrophic pathogen infection.

It is well-known that the plant hormone SA acts in defense signaling in plants (Ding et al. 2018; Fu et al. 2012; Zhao et al. 2019). SA signaling is transduced by the SA receptor, non-expressor of genes *PR1* (NPR1). NPR1 interacts with TGA transcription factors in the nucleus and stimulates their DNA

Fig. 2. Ca²⁺ enhanced the resistance of pear leaves to *Botryosphaeria dothidea* infection. **A**, Phenotypes of H₂O- and Ca²⁺-treated leaves under mock control and inoculation conditions at 10 days after inoculation (dpi). **B**, The calcium concentrations in H₂O- and Ca²⁺-treated leaves under mock control conditions at 6 dpi. **C**, The diameter of disease spots of leaves treated with Ca²⁺ at 10 dpi. Data are means of three replicates with standard error. Asterisks denote statistically significant differences between Ca²⁺- and H₂O-treated leaves (asterisks [**] mean $P < 0.01$, analysis of variance).

binding activity (Dong 2004). TGA transcription factors bind to cognate elements in promoters of certain genes and enhance their expression including *PR1* (*PATHOGENESIS-RELATED 1*) and *PR5*. Recently, it has been reported that SA is involved in plant resistance to *Botryosphaeria dothidea*. SA improved the tolerance of apple to *B. dothidea* by increasing *MdPR4* expression (Bai et al. 2013). Silencing *MdSYP121* resulted in enhanced resistance to *B. dothidea* by increasing SA levels (He et al. 2018). Zhao et al. (2019) found that *MdWRKY15* improves tolerance to *B. dothidea* by increasing SA synthesis by binding the *MdICS1* promoter in apple. Interestingly, studies have reported that Ca^{2+} signaling is connected to the SA-mediated immune response in *Arabidopsis* (Coca and San Segundo 2010; Du et al. 2009; Wang et al. 2009).

B. dothidea, a necrotrophic pathogen, causes pear ring rot in leaf, branch, and fruit, which is one of the most serious diseases in pear (*Pyrus bretschneideri* Rehd.) (Kim et al. 2001). However, the resistance mechanism of pear to *B. dothidea* is not clear. In this study, we used exogenous calcium treatment to simulate the increase of calcium concentration in pear leaves. The calcium-treated leaves showed enhanced resistance to *B. dothidea* infection, probably resulting from the decreased H_2O_2 and O_2^- levels. The Ca^{2+} treatment stimulated stronger enzymatic and nonenzymatic antioxidant under pathogen infection condition. Moreover, the activity of autophagosomes was higher, and SA was accumulated in the Ca^{2+} -treated pear leaves under inoculation. The expression levels of core ATG and SA-related genes were induced to higher levels in

Fig. 3. Changes in levels of reactive oxygen species (ROS) accumulation and activity of ROS-scavenging enzymes in pear leaves under *Botryosphaeria dothidea* infection. **A**, Quantitative measurement of H_2O_2 and **B**, O_2^- concentrations of pear leaves with and without Ca^{2+} treatment. **C**, Activity of superoxide dismutase (SOD), **D**, peroxidase (POD), **E**, catalase (CAT) and **F**, polyphenol oxidase (PPO) at 10 days after inoculation. Data are means of three replicates with standard error. Values not followed by the same letter indicate significant differences between treatments, according to one-way analysis of variance Tukey's multiple range tests ($P < 0.05$).

Ca²⁺-treated leaves under *B. dothidea* infection. These results demonstrated that calcium plays a key role in the resistance of pear to *B. dothidea* by enhancing autophagic activity and SA levels.

RESULTS

Effect of different calcium concentrations on pear resistance to *B. dothidea*.

Previous studies revealed that exogenous calcium promotes the senescence of broccoli microgreens and browning of pear fruit (Kou et al. 2014, 2015). Here, we applied different concentrations of CaCl₂ treatment on Dangshan Su pear (*Pyrus bretschneideri* Rehd.) leaves. As shown in Figure 1, treatment with 0.5 to 4% CaCl₂ significantly improved resistance to *B. dothidea*. As the Ca²⁺ concentration increased (0.5 to 4%), the diameter of disease spot increased (Fig. 1). At these concentrations, the effect of 1% Ca²⁺ treatment was the most significant. The diameter of the disease spot of 1% Ca²⁺-treated leaves was 34.98% that of H₂O-treated leaves. However, the

diameter of the disease spot of 4% Ca²⁺-treated leaves was slightly higher but not significantly higher than that of 3% Ca²⁺-treated leaves.

Phenotype of 1% Ca²⁺-treated leaves and endogenous Ca²⁺ content.

As shown in Figure 1, the effect of 1% Ca²⁺ treatment was the most significant among different Ca²⁺ concentrations. Therefore, we chose 1% Ca²⁺ to further investigation. The results were consistent with Figure 1, where 1% Ca²⁺ treatment significantly enhanced the resistance of pear to *B. dothidea*. The diameter of disease spots of calcium treated leaves was significantly lower than that of H₂O treated leaves (Fig. 2C). To examine whether exogenous Ca²⁺ increased the total calcium content, we also detected the total calcium content in pear leaves. As shown in Figure 2, at 6 days after inoculation (dpi), the calcium content in the treated leaves was significantly higher than that in the control leaves. The calcium content of treated leaves was 19.6% higher than that of control leaves.

Fig. 4. Changes in antioxidant concentrations of pear leaves and in transcript levels of genes implicated in the AsA-GSH cycle under inoculation. Concentrations of **A**, ascorbic acid (AsA), **B**, dehydroascorbate (DHA), **C**, AsA+DHA, and **D**, AsA/DHA. **E**, Concentrations of glutathione (GSH), **F**, GSH oxidized (GSSG), **G**, GSH+GSSG, and **H**, GSH/GSSG. These data were measured at 10 days after inoculation (dpi). **I**, Changes in expression of *PbrAPX*, **J**, *PbrDHR2*, **K**, *PbrMDHAR*, and **L**, *PbrGR* under inoculation. Total RNA was isolated from leaf samples collected at 6 dpi. Expression in H₂O-treated leaves under control conditions was set to “1”. Data are means of three replicates with standard error. Values not followed by the same letter indicate significant differences between treatments, according to one-way analysis of variance Tukey’s multiple range tests ($P < 0.05$).

Changes in activity of enzyme antioxidative system after calcium treatment under *B. dothidea* infection.

It is well-established that reactive oxygen species (ROS), also known as an oxidative burst, has been considered associated with plant resistance to pathogen infection (Lamb and Dixon 1997). To analyze the oxidative status after calcium treatment under *B. dothidea* infection, we examined the contents of H_2O_2 and O_2^- , two major versatile and stable ROS. H_2O_2 and O_2^- were induced in all leaves after inoculation. However, compared with H_2O treatment, Ca^{2+} treatment significantly reduced ROS production. The H_2O_2 and O_2^- contents of Ca^{2+} treated leaves were 19.63 and 21.90% lower than those of H_2O treated leaves, respectively (Fig. 3).

We also analyzed antioxidant enzymes after inoculation. The superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and polyphenol oxidase (PPO) activities did not differ significantly between calcium-treated and control leaves

(Fig. 3). After inoculation, the SOD, POD, CAT, and PPO levels were elevated, but the activities of these enzymes were significantly higher in Ca^{2+} -treated leaves than in H_2O -treated leaves.

Changes in the ascorbic acid-glutathione (AsA-GSH) cycle after calcium treatment under *B. dothidea* infection.

The AsA-GSH cycle is a nonenzyme system involved in scavenging ROS (Conklin 2001; Gallie 2013; Sun et al. 2018b). Here, we detected the status of AsA, dehydroascorbate (DHA), GSH, and oxidized GSH (GSSG) after Ca^{2+} treatment. *B. dothidea* infection significantly increased the AsA and DHA contents (Fig. 4A and B). The AsA level in Ca^{2+} -treated leaves was 2.04 times that in H_2O -treated leaves (Fig. 4A). However, the DHA level did not differ between Ca^{2+} - and H_2O -treated leaves (Fig. 4B). The total ascorbate (AsA+DHA) level in Ca^{2+} -treated leaves was significantly higher than that in H_2O -treated

Fig. 5. Changes in expression of core ATGs of pear leaves under inoculation. Total RNA was isolated from leaf samples collected at 6 days after inoculation. Expression in H_2O -treated leaves under control conditions was set to "1". Data are means of three replicates with standard error. Values not followed by the same letter indicate significant differences between treatments, according to one-way analysis of variance Tukey's multiple range tests ($P < 0.05$).

leaves under inoculation (Fig. 4C). This difference was more remarkable for the ratio of AsA to DHA (Fig. 4D). A consistent pattern was detected for the levels of GSH, GSSG, and the total GSH pool (Fig. 4E to G). For example, GSH content was obviously higher in Ca^{2+} -treated leaves than in H_2O -treated leaves (Fig. 4E). The ratio of GSH to GSSG was much higher in Ca^{2+} -treated leaves (Fig. 4G).

We also analyzed the expression levels of *APX*, *DHAR2*, *MDHAR*, and *GR*, four major genes involved in the AsA-GSH cycle. A similar change was observed in the levels and status of AsA and GSH. The expression levels of *APX*, *DHAR2*, *MDHAR*, and *GR* were induced by pathogen infection at 6 dpi, especially in the Ca^{2+} -treated leaves. For instance, the expression level of *APX* in Ca^{2+} -treated leaves was 2.53 times that of H_2O -treated leaves under inoculation conditions (Fig. 4I). These results indicate that Ca^{2+} treatment improved the capacity to produce and to maintain higher levels of beneficial antioxidants under pathogen infection.

Changes in the expression levels of core ATG genes and autophagic activity after calcium treatment under *B. dothidea* infection.

To explore the effect of Ca^{2+} treatment on autophagic activity under infection conditions, we first analyzed the expression levels of core ATG genes. Under mock control conditions, the transcript levels of *PbrATG1*, *PbrATG5*, *PbrATG6*, *PbrATG7*, *PbrATG8c*, and *PbrATG10* did not differ between the H_2O and Ca^{2+} treatment groups (Fig. 5). However, at 6 dpi, the expression levels of all genes were significantly higher in the Ca^{2+} -treated leaves than in the H_2O -treated leaves.

To investigate the autophagy occurrence in response to Ca^{2+} treatment under pathogen infection, we used transmission electron microscopy (TEM) to detect the numbers of autophagosomes. As shown in Figure 6A, few autophagosomes were observed in all treated leaves under mock control conditions. When infected by *B. dothidea*, the number of autophagosomes and autophagic bodies that accumulated in Ca^{2+} -treated leaf cells were higher than in H_2O -treated leaf cells (Fig. 6A and B). These results suggest that Ca^{2+} treatment increases autophagosome formation to enhance resistance to pathogen infection.

Calcium enhances resistance to *B. dothidea* in pear plants in an autophagy-dependent manner.

To deeply explore the mechanism by which calcium affects the resistance of pear plants to *B. dothidea*, we performed the virus-induced gene silencing (VIGS) of a key gene in autophagy,

PbrATG5, in *P. betulaefolia*. As shown in Figure 7A, the expression level of *PbrATG5* was significantly lower in TRV2-*PbrATG5* pear plants than in TRV2 plants. Silencing of *PbrATG5* significantly increased the disease diameter under H_2O treatment (Fig. 7B). However, the calcium treatment produced only a slight recovery of the symptoms of inoculation in TRV2-*PbrATG5* pear plants (reduced by 14.2%) compared with TRV2 control plants (reduced by 58.8%) (Fig. 7C). These results indicated that calcium enhance the resistance of pear plants, at least in part, through autophagy.

Changes in SA accumulation and expression levels of SA-related genes after calcium treatment under *B. dothidea* infection.

Many articles have reported that SA plays a role in the plant immunity response (Dong 2004; Fu et al. 2012; Hofius et al. 2017; Sun et al. 2018b; Zhao et al. 2019). To explore SA synthesis in response to Ca^{2+} treatment, we detected SA concentrations. In Figure 8A, the SA level was induced by Ca^{2+} under pathogen infection. For the inoculation, the SA content was 1.83 times higher in the Ca^{2+} -treated leaves than in the H_2O -treated leaves. Two SA synthesis genes, *ICS1* and *EDS1*, were more highly expressed in Ca^{2+} -treated leaves than in H_2O -treated leaves, whereas the transcription of these genes was not different under the mock control condition. Furthermore, the expression levels of three SA-related genes, *NPR1*, *PR1*, and *PR5*, were higher in the Ca^{2+} -treated leaves than in the H_2O -treated leaves. In addition, expression of genes involved in the jasmonic acid (JA) and ethylene pathway were also examined. As shown in Supplementary Figure S1, the expression of the JA-related genes *PbrPR3* and *PbrPR4* was significantly reduced in Ca^{2+} -treated leaves under inoculation. The expression levels of two ethylene synthesis genes that have been studied previously, *PbrACS1B* (Shi et al. 2013) and *PbrACO54* (Hao et al. 2017), were also examined. As shown in Supplementary Figure S1, the expression of *PbrACS1B* and *PbrACO54* was induced by *Botryosphaeria dothidea*. However, the expression levels of *PbrACS1B* and *PbrACO54* were lower after calcium treatment than after H_2O treatment under inoculation. These results indicated that calcium treatment enhanced SA-related gene expression.

DISCUSSION

Calcium has been reported to participate in plant metabolism (Kou et al. 2014, 2015; Yuan et al. 2017). Previous studies

Fig. 6. Accumulation of autophagosomes under inoculation. **A**, Representative transmission electron microscopy images of autophagic structures in mesophyll cells from H_2O - and Ca^{2+} -treated leaves. Bars = 1 μm . **B**, Relative autophagic activity normalized to the activity of H_2O - and Ca^{2+} -treated leaves shown in A. More than 10 cells were used to quantify structures. Data are means of three replicates with standard error. Values not followed by the same letter indicate significant differences between treatments, according to one-way analysis of variance Tukey's multiple range tests ($P < 0.05$).

Fig. 7. Silencing of *PbrATG5* decreased the resistance to *Botryosphaeria dothidea* and led to only a slight recovery by calcium treatment. **A**, Phenotype of TRV2 and TRV2-*PbrATG5* leaves infected by *B. dothidea* under calcium and H₂O treatment at 6 days after inoculation. **B**, The expression level of *PbrATG5* in *Pyrus betulaefolia*. **C**, Diameter of disease spots of *PbrATG5*-silenced plants. Data are means of three replicates with standard error. Asterisks denote statistically significant differences between TRV2 and TRV2-*PbrATG5* plants (asterisks [**] mean $P < 0.01$, analysis of variance).

found that exogenous calcium treatment could promote plant senescence, yield, fruit browning, and abiotic tolerance (Kou et al. 2014, 2015; Yin et al. 2015). However, little is known about the effect and mechanism of exogenous calcium application in plant immunity. Here, we sprayed 1% CaCl₂ on pear leaves to detect resistance to *B. dothidea* infection. We found that exogenous Ca²⁺ treatment significantly enhanced resistance to *B. dothidea*. The Ca²⁺ treatment increased the total calcium concentrations in the pear leaves. The Ca²⁺-treated leaves accumulated fewer ROS and showed enhanced activities of ROS-scavenging enzymes and capacity for AsA-GSH recycling under inoculation. Most importantly, under inoculation conditions, the activity of autophagy and the level of SA was significantly higher in the Ca²⁺-treated leaves than in the H₂O-treated leaves. These results suggested that exogenous Ca²⁺ treatment enhanced resistance to infection by *B. dothidea*.

Previous studies found that appropriate Ca²⁺ concentrations could benefit plant development and adaptation to stress, but excessive calcium application may impair normal metabolism in plant (Kou et al. 2014). Our results indicate that the effect of exogenous calcium on resistance to *B. dothidea* is dose-dependent. The 1% CaCl₂ treatment resulted in the best resistance. However, high doses of calcium application (2 to 4% CaCl₂) showed a decreased effect on resistance. As the second messenger, calcium is involved in multiple cellular processes via transmitting signals received at the cell surface to the interior by changes in cytosol concentrations, which are decoded by a series of Ca²⁺ sensors (Kudla et al. 2010; Ranty et al. 2016; Yang and Poovaiah 2003). Under normal conditions, intracellular calcium concentration can be well controlled by calcium influx and efflux mechanisms in the cell membrane; however, the excessive application of calcium affects the balance of calcium influx and efflux, resulting in disordered intracellular calcium (Kudla et al. 2010; Steinhorst and Kudla 2014). These uncontrolled changes in calcium concentration ultimately lead to cell damage. Further research is needed to explore the molecular mechanisms underlying the role of excessive calcium in resistance to pathogen infection.

The ROS stimulated by pathogens are highly reactive and, therefore, toxic and participate in several important processes related to defense and infection (Shetty et al. 2008). H₂O₂ and O₂⁻ are two major and stable ROS. In our study, the content of H₂O₂ and O₂⁻ was significantly lower in the Ca²⁺-treated leaves than in the H₂O-treated leaves under inoculation conditions. This result was consistent with a previous study that reported lower ROS accumulation in more resistant apple plants under pathogen infection (Sun et al. 2018b; Zhao et al. 2019). Plants utilize both enzymatic and nonenzymatic mechanisms to scavenge ROS (Cheng et al. 2016; Sun et al. 2018b; Xing et al. 2019). Here, the activities of SOD and POD were significantly higher in Ca²⁺-treated leaves under inoculation. Similarly, as the nonenzymatic system for scavenging ROS, the AsA-GSH cycle was maintained at a reduced status by Ca²⁺ treatment under inoculation. Moreover, four genes located in different cellular compartments that work in conjunction with the AsA-GSH cycle, namely, *APX*, *DHAR*, *MDHAR*, and *GR*, were expressed at higher levels in the Ca²⁺-treated leaves than in the H₂O-treated leaves under *B. dothidea* infection. Previous studies indicated that enhanced autophagy activity led to the modification of the antioxidant system (Sun et al. 2018a and b). Thus, the higher antioxidative status in the Ca²⁺-treated leaves may result from the higher activity of autophagy.

Numerous studies in mammals have reported that calcium stimulates autophagy by calcium sensors (East and Campanella 2013; Kondratskyi et al. 2018; La Rovere et al. 2016). In *Arabidopsis*, CML24 affects autophagy progression by interacting

with ATG4 (Tsai et al. 2013). Autophagy deficiency in *Arabidopsis* mutants showed spreading necrotic lesions and enhanced fungal growth upon infection with necrotrophic pathogen (Lai et al. 2011; Lenz et al. 2011). These results indicated that autophagy enhances plant resistance to necrotrophic pathogens. In our study, the Ca^{2+} -treated pear leaves accumulated more autophagosomes than did H_2O -treated leaves under inoculation. Moreover, the expression levels of core ATG genes was significantly increased in Ca^{2+} -treated leaves compared with H_2O -treated leaves under *B. dothidea* infection. The resistance of *PbrATG5*-silenced pear leaves was decreased and calcium treatment only slightly restricted disease spots under inoculation. These results suggested that the increased autophagy activity was attributed to the enhanced resistance to *B. dothidea*. Our discoveries provide evidence that autophagy could be activated by Ca^{2+} .

Previous studies indicated that a mechanism connects Ca^{2+} signals to the SA-mediated immune response (Coca and San Segundo 2010; Du et al. 2009; Wang et al. 2009). SA is involved in both local defense responses at the infection site and the activation of systemic resistance (Robert-Seilanianz et al. 2011). CBP60g contributes to microbe-associated molecular pattern-triggered SA accumulation in *Arabidopsis* (Wang et al. 2009). AtCPK1 enhances the resistance of *Arabidopsis* to pathogens by regulating the accumulation of SA and the expression of SA-related genes (Coca and San Segundo 2010). These results indicated that SA content is an important factor in the regulation of plant resistance to pathogens. Here, the Ca^{2+} -treated leaves accumulated higher levels of SA than did the H_2O -treated leaves. It was demonstrated that exogenous Ca^{2+} increased SA synthesis. Two SA synthesis genes, *ICS1* and *EDS1*, were more strongly induced in the Ca^{2+} -treated leaves

Fig. 8. Changes in salicylic acid (SA) synthesis and expression of SA-related genes. **A**, SA content was measured at 10 days after inoculation (dpi). **B** through **F**, Expression of *PbrNPR1*, *PbrEDS1*, *PbrNPR1*, *PbrPR1*, and *PbrPR5* was analyzed at 6 dpi. Expression in H_2O -treated leaves under control conditions was set to "1". Data are means of three replicates with standard error. Values not followed by the same letter indicate significant differences between treatments, according to one-way analysis of variance Tukey's multiple range tests ($P < 0.05$).

after inoculation. SA signaling was transduced by the *NPR1* gene and elevated *PR* gene expression to enhance disease resistance (Ding et al. 2018; Hussain et al. 2018). We noted here that the expression levels of *PbrNPR1*, *PbrPR1*, and *PbrPR5* were elevated by Ca^{2+} treatment compared with H_2O treatment. These results demonstrated that Ca^{2+} regulated the SA pathway and contributed to pathogen resistance.

In conclusion, exogenous calcium treatment enhanced resistance to *B. dothidea* by increasing autophagic activity and SA level in pear. The Ca^{2+} -treated pear leaves accumulated lower levels of ROS and showed higher SOD and POD activity. The AsA-GSH cycle was maintained at a more-reductive redox state by increasing the total calcium content. This might result from by enhanced autophagic activity, as supported by the elevated expression of core *PbrATG* genes and the increase in autophagosome formation. The SA pathway was activated more by Ca^{2+} treatment than by H_2O treatment under inoculation. These results provide further insight into the function of calcium in pear disease resistance. We can use the strategy of exogenous calcium spraying on the leaf, which could induce autophagy and SA to alleviate pathogen infections according to our results.

MATERIALS AND METHODS

Plant materials and treatments.

Healthy mature leaves of Dangshan Su pear (*Pyrus bretschneideri* Rehd.), obtained from the 'Hu Shu' pear orchard of Nanjing Agricultural University, were detached with their complete petioles and were immediately taken to the laboratory. After the leaves were prepared and surface-sterilized with 0.1% sodium hypochlorite for 10 min, the preprocessed leaves were washed with sterile water three times, and the petioles were cut short underwater, to prevent gas bolting and were wrapped with wet absorbent gauze. The leaves were then sprayed with 0.5 to 4% CaCl_2 and double-distilled H_2O solutions containing 0.05% Tween-20. After 12 h, the leaves were wounded at four locations, using a sterile needle. The wounded samples were inoculated with mycelium agar (5 mm diameter) obtained from the edge of an activated colony of *B. dothidea* on potato dextrose agar (PDA) or control PDA agar. They were then incubated in the dark in a chamber held at a constant 25°C and 80% relative humidity. A total of 180 leaves in four plastic boxes were inoculated or not inoculated under CaCl_2 and H_2O treatment, with four inoculation sites per leaf. Five entire leaves were sampled per repeat at 0, 6, and 10 dpi. Leaves were sampled three times as three repeats with a total of 15 leaves at each sampling time.

Total calcium content detection.

The total calcium content was measured as described previously (Codling et al. 2007; Kou et al. 2014). Briefly, at 6 dpi, the entire leaves were rinsed three times with double distilled water to remove any surface calcium before drying in a 70°C oven for 48 h. The over-dried leaf tissues (0.5 g) were ashed at 550°C for 16 h, followed by the addition of 5 ml of concentrated nitric acid; samples were then dried on a hot plate. After drying, the digest volumes were brought to 25 ml with 0.1 M HCl. The calcium concentration in the tissue was determined using the Optima 4300 DV inductively coupled plasma optical emission spectrometer (PerkinElmer), with strontium as an internal standard.

Detection of H_2O_2 , O_2^- , and antioxidant metabolites.

The H_2O_2 , O_2^- , CAT, PPO, POD, SOD, AsA, DHA, GSH, and GSSG detection assays were performed according to manufacturer instructions (Comin).

RNA extraction and real-time PCR.

Total RNA was extracted according to a CTAB method. First-strand cDNA synthesis and real-time PCR were performed as previously described (Xing et al. 2019). *Tubulin* was used as internal control. Primer sequences for real-time PCR are listed in Supplementary Table S1. Each experiment was repeated three times biologically, based on three separate RNA extracts from three repeats.

Autophagosome detection by TEM.

At 6 dpi, small pieces of mesophyll located at the border of the disease spot were cut from the leaves and, then, were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffered saline (pH 7.4) before being placed in the dark for 12 h at 4°C. Our TEM analysis was performed as previously described (Sun et al. 2018a).

Vector construction and plant transformation.

Tobacco rattle virus-based vector (pTRV1/2) constructs were used for VIGS (Xing et al. 2019). To construct of pTRV-*PbrATG5*, a fragment of the *PbrATG5* open reading frame (306 bp, 420 to 725 bp) was amplified by PCR, which was then introduced into the pTRV2 vector. The primer was shown in Supplementary Table S1. VIGS of *PbrATG5* was performed as previously described (Xing et al. 2019).

Detection of SA content.

SA was extracted and quantified as described previously (Fu et al. 2012; Sun et al. 2018b). Leaf samples (0.1 g) were homogenized in liquid nitrogen and were placed in 1 ml of 90% methanol. 3-Hydroxybenzoic acid (Sigma) was added as internal standard. Extracts were analyzed through a fluorescence detector (excitation at 305 nm and emission at 405 nm) on a Zorbax SB-C18 column (Agilent Technologies).

Statistical analysis.

Experimental data are presented as the means $\pm$ standard deviations of three independent replicates. Data were analyzed via analysis of variance, and mean values were compared by Tukey's multiple range test ($P < 0.05$). All the statistical analyses were performed using SPSS18 statistical software package (IBM SPSS Statistics).

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