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Research article

Salicylic acid confers enhanced resistance to Glomerella leaf spot in apple

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ABSTRACT

Glomerella leaf spot (GLS) caused by *Glomerella cingulata* is a newly emergent disease that results in severe defoliation and fruit spots in apple. Currently, there are no effective means to control this disease except for the traditional fungicide sprays. Induced resistance by elicitors against pathogens infection is a widely accepted eco-friendly strategy. In the present study, we investigated whether exogenous application of salicylic acid (SA) could improve resistance to GLS in a highly susceptible apple cultivar (*Malus domestica* Borkh. cv. 'Gala') and the underlying mechanisms. The results showed that pretreatment with SA, at 0.1–1.0 mM, induced strong resistance against GLS in 'Gala' apple leaves, with SA treated leaves showing significant reduction in lesion numbers and disease index. Concurrent with the enhanced disease resistance, SA treatment markedly increased the total antioxidant capacity (T-AOC) and defence-related enzyme activities, including catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO). As expected, SA treatment also induced the expression levels of five pathogenesis-related (*PR*) genes including *PR1*, *PR5*, *PR8*, *Chitinase* and *β-1,3-glucanase*. Furthermore, the most pronounced and/or rapid increase was observed in leaves treated with SA and subsequently inoculated with *G. cingulata* compared to the treatment with SA or inoculation with the pathogen. Together, these results suggest that exogenous SA triggered increase in reactive oxygen species levels and the antioxidant system might be responsible for enhanced resistance against *G. cingulata* in 'Gala' apple leaves.

1. Introduction

Glomerella leaf spot (GLS) of apple caused by *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk is an emerging disease and has become a serious problem in China (Wang et al., 2012, 2015a). In China, GLS was first noticed in August 2011 in Fei-xian, Jiangsu Province (Wang et al., 2012), and currently, the disease has disseminated almost all of the apple producing areas (Wang et al., 2015a). It has been observed that 'Gala' and other important commercial cultivars in China, such as 'Qinguan' and 'Golden Delicious' are highly susceptible to GLS (Wang et al., 2015a). The pathogen mainly infects apple leaves and fruits but can also attack twigs. Under favorable conditions, GLS can result in more than 90% defoliation and diseased fruits in China, thereby reducing fruits yield and quality and weakening apple tree vigor (Wang et al., 2015a). On leaves, the disease is first manifested by lots of small black spots and these expand rapidly causing premature defoliation within a few weeks (Wang et al., 2012; Moreira and May De Mio, 2015). Infected fruits show many small brown to black lesions that range from 1 to 5 mm in diameter.

Conidia produced by *G. cingulata* are the primary infection source during early stages of disease epidemics. High humidity and a temperature between 20 and 30 °C are typically favorable for conidial germination, infection, and disease development (Wang et al., 2015a). Under these conditions, a large number of conidia are produced on diseased leaves within 3–5 days and disseminated by wind-blown rain. Due to the short incubation period, it is particularly difficult to control GLS after the pathogen infection (Wang et al., 2012, 2015a).

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Up to now, control of GLS is based on the preventive applications of fungicides (Becker et al., 2000; Wang et al., 2015a). However, issues associated with environmental and human health, fungicide resistance and increased production costs have motivated the development of eco-friendly approaches for plant protection. The application of elicitors activates natural resistance in plants against pathogens infection and is considered as a promising alternative strategy to traditional chemical treatments (Tian et al., 2006; Yu et al., 2014). Salicylic acid (SA) is one of the chemical elicitor that has been reported to induce resistance against multiple fungal, viral and bacterial pathogens in a variety of crop plants (Chen et al., 2006; Radwan et al., 2010; Czajkowski et al., 2015). SA is known to increase the activities of phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO), which participate in the synthesis of phenolic compounds and strengthen the cell wall at sites of infection (Tian et al., 2006; Mandal et al., 2009). Previously studies have demonstrated that SA treatment induces PAL and PPO activities in crop plants and confers resistance against fungal pathogens (Chen et al., 2006; Tian et al., 2006; Cao et al., 2013).

Exogenous SA is thought to regulate the antioxidant system in apple, sweet cherry and faba bean (Xu and Tian, 2008; Radwan et al., 2010; Zhong et al., 2013). Reactive oxygen species (ROS) often accumulate in plants in response to pathogen infection and the pivotal role of ROS in the regulation of plant defence response is well established (Torres et al., 2006; Wang et al., 2014). However, excessive accumulation of ROS can cause oxidative damage and induce membrane lipid peroxidation in the cellular environment (Radwan et al., 2010). ROS-scavenging systems play an important role in regulating the amount of ROS that is produced in plant cells. The main scavenging mechanism includes enzymatic antioxidants and metabolites (Mittler, 2002; Foyer and Noctor, 2005). The major antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). SOD catalyzes the dismutation of O_2^- to O_2 and H_2O_2, which is further converted to water and oxygen by CAT and/or POD (Radwan et al., 2010).

Various studies have been demonstrated that the final outcome of the induced defence responses is the potentiated expression and accumulation of pathogenesis-related proteins (PRs) (Jones and Dangl, 2006). Some of these PRs, such as chitinase (PR3) and β-1,3-glucanase (PR2), downstream components of defence signaling, possess direct antimicrobial activities and participate in antifungal defence (Mauch et al., 1988). In apple leaves, chitinase and β-1,3-glucanase are induced in response to *Diplocarpon mali* infection (Yin et al., 2013b) and the experimental evidence suggests that the two PRs increase in response to exogenous SA in jujube (Cao et al., 2013) or treatment with γ-aminobutyric acid (GABA) in pear fruit (Yu et al., 2014). Moreover, Zhang et al. (2012) showed that *PR8* expression in *Malus hupehensis* leaves can be induced by SA and *Botryosphaeria berengeriana* infection. Liu et al. (2013) suggested that *PR8* expression in apple fruit is associated with the response to *Botrytis cinerea* infection, and may play a role in yeast induced resistance against *B. cinerea*. *PR5*, a thaumatin-like protein, has been shown to have antifungal activity (Vigers et al., 1992). Both *PR1* and *PR5* are widely used as molecular markers that correlate with accumulation of endogenous SA (Durrant and Dong, 2004).

The induced resistance conferred by exogenous SA has been investigated using different plant-fungal pathosystems. However, most of these studies were carried out using model plant systems such as *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) (Edgar et al., 2006; Mandal et al., 2009). To our knowledge, so far no information is available concerning exogenous SA to enhance apple resistance to GLS caused by *G. cingulate*.

The aim of this study was to determine whether exogenous application of SA could improve resistance in susceptible 'Gala' apple to GLS and investigate the underlying mechanisms. We monitored the antioxidant response and activity changes of PAL and PPO in 'Gala' apple leaves. In addition, we also examined the expression of a set of pathogenesis-related genes in SA defence signaling pathways or encoding the proteins participating in antifungal defence by quantitative real-time PCR (RT-qPCR).

2. Materials and methods

2.1. Plant material and inoculum preparation

The current study used the plants of *Malus domestica* Borkh. cv. 'Gala', a susceptible cultivar, which is the most widely cultivated apple in China. For the experiments, the three-year-old apple trees on the M9T337 rootstock were grown in a greenhouse, Qingdao Agricultural University, Qingdao, Shandong Province, China. Standard horticultural practices were carried out.

A monospore culture 0101 of *G. cingulata* was isolated from a diseased 'Gala' apple leaf showing GLS symptoms which was sampled from an orchard in Laixi, Shandong Province, China (Wang et al., 2015a). The isolate was maintained on potato dextrose agar (PDA: the extract of 200 g potato, 20 g of glucose and 15 g of agar in 1000 mL water) at 4 °C. The conidia were produced on PDA according to the method described by Wang et al. (2015a). Conidial suspension used for the in vitro germination assays were counted using hemocytometer and adjusted to 1×10^6 conidia mL^{-1}. A concentration of 5×10^4 conidia mL^{-1} was used for inoculations of apple leaves.

2.2. Effect of SA at different concentrations on conidial germination and mycelial growth of G. cingulata in vitro

SA (Sigma, St. Louis, MO, USA) stock solution was prepared in sterile distilled water. The effect of SA on conidial germination of *G. cingulata* was tested with various concentrations (0, 0.1, 0.2, 0.5 and 1.0 mM). Aliquots of 200 μL of conidial suspensions were put onto concave slides to obtain a final concentration of 1×10^6 conidia mL^{-1}. After 12 h of incubation at 25 °C, approximately 150–200 conidia of the pathogen per replicate were measured for germination rate. The conidia were considered germinated when the germ tube length was longer than half of the total length of the conidia. All treatments consisted of four replicates and the experiment was repeated thrice.

The effect of SA on mycelial growth of *G. cingulate* was evaluated on PDA plates according to the method of Cao et al. (2013) with some modifications. A 5-mm *G. cingulate* agar disk from actively growing mycelium of the pathogen was transferred into a new PDA plate with different concentrations of SA (0, 0.1, 0.2, 0.5 and 1.0 mM). The mycelial disk was placed in the center of the plate (90 mm in diameter). After 96 h of incubation at 25 °C, the colony diameters were measured by the cross method. Each of the SA concentrations was replicated on four plates and the experiment was repeated thrice.

2.3. Induction of 'Gala' apple resistance against GLS by SA at different concentrations

'Gala' apple trees of uniform size were selected to determine the proper concentration of SA that could effectively induce disease resistance against *G. cingulate* infection. The trees were sprayed with 0 (control), 0.1, 0.2, 0.5, 1.0 mM concentrations of SA solution. After 2 days of the treatment, shoots with fully expanded and healthy leaves were selected and detached from the trees. The shoots with 3–4 leaves that were similar in size were then inoculated by spraying the conidial suspensions (5×10^4 conidia mL^{-1}) of *G. cingulate* on the leaves (Wang et al., 2015a). The shoots were