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Chitosan and oligochitosan enhance the resistance of peach fruit to brown rot

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ABSTRACT

The effects of chitosan and oligachitosan on resistance induction of peach fruit against brown rot caused by *Monilinia fructicola* were investigated. Both chitosan and oligochitosan showed significant effect on controlling this disease. Moreover, chitosan and oligochitosan delayed fruit softening and senescence. The two antifungal substances enhanced antioxidant and defense-related enzymes, such as catalase (CAT), peroxidase (POD), β-1,3-glucanase (GLU) and chitinase (CHI), and they also stimulated the transcript expression of *POD* and *GLU*. These findings suggest that the effects of chitosan and oligochitosan on disease control and quality maintenance of peach fruit may be associated with their antioxidant property and the elicitation of defense responses in fruit.

1. Introduction

Peach fruit has a short postharvest life at room temperature due to rapid quality deterioration and high susceptibility to pathogens (Sasaki et al., 2010). Brown rot caused by *Monilinia fructicola* is a major disease on peach fruit (Zhou, Schneider, & Li, 2008). Although synthetic fungicide treatment has been the main method for controlling postharvest diseases, growing public concern over the potential impact of synthetic fungicides on environment and human health has created interest in exploring new alternatives for the disease management (Bautista-Baños et al., 2006; Droby, Wisniewski, Macarisin, & Wilson, 2009).

As promising alternatives, chitosan and its derivatives like oligochitosan, have been reported to effectively control postharvest diseases. Chitosan, the linear and partly acetylated (1-4)-2-amino-2-deoxy-β-D-glucan, is obtained from marine chitin, the second most abundant carbohydrate polymer after cellulose (Muzzarelli et al., 2012). Oligochitosan was obtained by hydrolysis or degradation of chitosan. Due to their chemical constitutions, chitosan and oligochitosan are cationic, nontoxic, biodegradable and biocompatible, but also possess versatile functional properties (Bautista-Baños et al., 2006; Kim & Rajapakse, 2005; Muzzarelli, 2010). In previous studies, they were reported to directly inhibit growth of some major fungal pathogens, such as *Botrytis cinerea* (Liu, Tian, Meng, & Xu, 2007), *Rhizopus stolonifer* (Hernández-Lauzardo et al., 2008), *Phytophthora capsici* (Xu, Zhao, Wang, Zhao, & Du, 2007), *M. fructicola* (Yang, Zhang, Bassett, & Meng, 2012), *Alternaria kikuchiana* and *Physalospora piricola* (Meng, Yang, Kennedy, & Tian, 2010). In addition of the direct antifungal effect, many researches focused on the potential of inducing defense response in fruit. Chitosan could boost the activity of defense-related enzymes or pertinent substance in tomato fruit (Liu et al., 2007), grape berry (Romanazzi, Nigro, Ippolito, Di Venere, & Salerno, 2002), strawberry and raspberry fruit (Zhang & Quantick, 1998). Likewise, oligochitosan has been shown to be effective to elicit production of hydrogen peroxide (Li, Yin, Wang, Zhao, Du, & Li, 2009; Lin, Hu, Zhang, Rogers, & Cai, 2005), to increase activity of phenylalanine ammonialyase (PAL) and peroxidase (POD) (Vander, Varum, Domard, Eddine El, & Moerschbacher, 1998), and to up-regulate gene expression of β-1,3-glucanase (GLU) and chitinase (CHI) (Lin et al., 2005). In addition of defense response, antioxidant response was also associated closely with fruit resistance against disease. Fungal pathogen infection can cause oxidative stress by inducing the generation of reactive oxygen species (ROS) in plants (Campo, Carrascal, Coca, Abián, & San Segundo, 2004). The undesirable oxidative stress can damage nucleic acids, lipids or protein, and thus inactivate key cellular functions (Levine, Wehr, Williams, Stadtman, & Shacter, 2000). Antioxidant response of fruit, therefore, is also one of major modes of action against pathogen infection (Xu, Qin, & Tian, 2008). Moreover, chitosan contributed positively to postharvest quality of various fruits, such as table grape (Meng, Li, Liu, & Tian, 2008) and citrus (Chien, Sheu, & Lin, 2007).

The overall objectives of this study were to (1) investigate the effects of chitosan and oligochitosan on peach fruit quality and on

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resistance induction to control brown rot caused by *M. fructicola*; (2) evaluate the activity and transcript expression of defense or antioxidant enzymes in peach fruit induced by chitosan or oligochitosan treatment.

2. Materials and methods

2.1. Fruit

Peach (*Prunus persica* (L.) Batsch) fruit were harvested at the mature stage and sorted based on the size and the absence of physical injuries or disease infection. Before treatments, fruit were disinfected on the surface with 2% (v/v) sodium hypochlorite for 2 min, then rinsed with tap water and air-dried (Liu et al., 2007). Then, the fruits were divided into five groups to dip in five different treatment solutions for 10 min and air-dried: (a) water (control), (b) 0. 5 g L^{-1} chitosan, (c) 5 g L^{-1} chitosan, (d) 0. 5 g L^{-1} oligochitosan, and (e) 5 g L^{-1} oligochitosan. The fruits in the five treatments were then divided into two lots, respectively:

Lot I (For investigation of disease incidence): in each group with 30 peach fruits, the fruits with 20 artificial wounds (4 mm deep and 3 mm wide) on its equator dipped with *M. fructicola* spore suspension at 1×10^4 spores mL^{-1} for 30 s, were used for investigation of disease incidence.

Lot II (For evaluation of fruit quality and response): In each group with 30 peach fruits, the fruits treated without wounding or dipping in *M. fructicola* spore suspension were for evaluation of fruit quality and response.

Treated fruits were placed in plastic box with a polyethylene bag and then stored at 25 °C. Each treatment contained three replicates with 30 fruits per replicate, and the experiment was repeated three times.

2.2. Determination of fruit quality

Flesh firmness was determined on opposite peeled cheeks of the fruit using a Fruit Firmness Tester (FT-327, Effegi, Alfonsine, Italy), equipped with an 8-mm plunger tip.

Titratable acidity (TA) was assayed according to Meng et al. (2008). Ten gram flesh tissue (about 5 mm deep under the peel) from 5 fruits on the transverse axis (each fruit on opposite regions) was homogenized with 25 mL distilled water. The homogenates were centrifuged by 17,000 × *g* for 30 min and the supernatants were used for TA assay. TA of the solution was determined by titration to pH 8.1 with 0.1 mol L^{-1} NaOH. TA is expressed as the concentration of H^+ per gram of fresh mass.

Determination of total soluble solids (TSS): juice samples were obtained from 10 discs of flesh (taken about 5 mm deep under the peel, 10 mm thickness × 10 mm diameter, 2 disks per fruit on opposite regions) from checks of 10 fruits. TSS of the fruit juice was determined using an Abbe Mark II refractometer (10481 S/N, Reichert, Buffalo, NY). Each treatment contained three replicates with 10 fruits per replicate, and the experiment was repeated three times.

2.3. Determination of activities in antioxidant and defense-related enzymes

The assay of POD was conducted following the method of Meng et al. (2008). Flesh tissues (10 g) from 10 fruits were collected and homogenized with 25 mL of ice-cold extraction buffer (100 mmol L^{-1} sodium phosphate, pH 6.4), containing 0.5 g of polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged (4 °C, 15,000 × *g*, 30 min) and the supernatants were analyzed immediately. For activity determination, 0.5 mL of enzyme in 2 mL buffer substrate (100 mmol L^{-1} sodium phosphate, pH 6.4 and 8 mmol L^{-1} guaiacol) was incubated for 5 min at 30 °C and the increase in absorbance at 460 nm for 2 min after adding 1 mL of H_2O_2 (24 mmol L^{-1}) was measured. The specific activity was expressed as U mg^{-1} protein, and the unit was defined as one ΔOD_{460} min^{-1} mg^{-1} protein.

CAT activity determination was performed according to the method of Zheng, Tian, Meng, & Li (2007) with some modification. Flesh tissues (10 g) from 10 fruits were collected and homogenized with 25 mL of ice-cold extraction buffer (50 mmol L^{-1} sodium phosphate, pH 7.0), containing 0.5 g of polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged (4 °C, 15,000 × *g*, 30 min) and the supernatants were analyzed immediately. For enzyme activity, 0.5 mL of enzyme in 2 mL buffer substrate was incubated for 5 min at 30 °C and the increase in absorbance at 460 nm for 2 min after adding 1 mL of H_2O_2 (24 mmol L^{-1}) was measured. The specific activity was expressed as U mg^{-1} protein, and the unit was defined as one ΔOD_{240} min^{-1} mg^{-1} protein.

For β-1,3-glucanase and chitinase activity assay, flesh tissues (10 g) from 10 fruits was mixed with 20 mL of sodium acetate buffer (50 mmol L^{-1}, pH 5.0) containing 0.5 g of polyvinyl polypyrrolidone (PVPP), and ground thoroughly at 4 °C. The homogenate was centrifuged (4 °C, 15,000 × *g*, 30 min). Activity of β-1,3-glucanase was determined, with laminarin as substrate, following the method described by Yao and Tian (2005), while chitinase was measured according to the method of Wirth and Wolf (1990) with chitin as a substrate, and reducing sugars as reaction production was measured spectrophotometrically at 550 nm and 500 nm using a UV-160 Spectrophotometer (Shimadzu, Japan). Specific activity of enzymes was expressed as U mg^{-1} protein, where one unit was defined as one μmol product h^{-1} mg^{-1} protein for chitinase and β-1,3-glucanase.

Protein content was determined according to Bradford (1976) with bovine serum albumin (Sigma Chemicals Co., St. Louis, USA) as standard. Each treatment contained three replicates and the experiment was repeated three times.

2.4. cDNA cloning

Total RNA was extracted from peach fruit at 0, 12, 24 and 48 h after treatment with 5 g L^{-1} chitosan or oligochitosan. First-strand cDNA was synthesized with first-strand cDNA synthesis kit and used for polymerase chain reaction (PCR). The highly conserved domains of peach *peroxidase* (*POD*) and *β*-1,3-glucanase (*GLU*) cDNA clones were obtained by PCR. All primers used were listed in Table 1. The PCR products were cloned into pGEM-T vector and sequenced.

2.5. Isolation of total RNA and northern blot analysis

About 10 g flesh tissue (about 2 mm away from the infection site or wound) from 10 fruits was ground in liquid nitrogen. Total RNA was extracted by the method hot-phenol isolation protocol and stored at −80°C. Northern blot analysis was conducted according to Xu et al. (2008). RNA (20 μg) was denatured in formamide and formaldehyde at 65°C and run in electrophoresis in a 1% agarose gel containing formaldehyde (6%, v/v). After electrophoresis, RNA was capillary transferred overnight onto a Hybond-N^+ membrane (Amersham International, Amersham, Bucks, UK) and hybridized with [^{32}P] dCTP-labeled cDNA probe. Pre-hybridization and hybridization were carried out at 65 °C. Blots were hybridized for 16 h and then washed once for 20 min in 2 × SSC, 0.1% SDS at 65 °C and twice for 10 min in 0.1 × SSC, 0.1% SDS at 65 °C. RNA bands were visualized by autoradiography. Equal loading of samples of total RNA was identified by visualization of rRNA that had

Table 1
Primers used in the cloning conserved domains of *POD* and *GLU* cDNA in peach fruit.

cDNA amplified	Primers	Temp. °C	Genbank accession
Peroxidase	F: cggtttggtgtactttgcgatcg R: tcatttattcatacagagctggc	60	DW353011, W350829 DW352089
β-1,3-glucanase	F: gtatgtaatggaatggttggcg R: tctcaagagcagcataaacacc	60	AF435088, AF435089 U49454

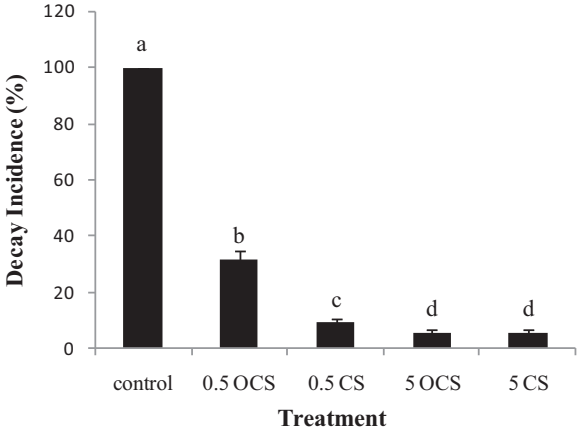

Fig. 1. Effect of chitosan and oligochitosan with different concentrations on diseases of peach fruit stored at 25 °C after 4 days. Bars represent standard deviations of the means according to three independent experiments.

been stained with ethidium bromide. Autoradiographs were digitally scanned. There were three replicates in each treatment, and the experiment was repeated three times. A representative image is presented in the manuscript.

2.6. Data analysis

All statistical analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way ANOVA. Mean separations were performed by Duncan's multiple range tests. Differences at $P < 0.05$ were considered significant. Data presented in this paper were pooled across three independent repeated experiments.

3. Results

3.1. Effects of chitosan and oligochitosan on control of brown rot in peach fruit

Disease incidence of brown rot in peach fruit caused by *M. fructicola* was investigated after 4 days of storage at 25 °C. As shown in Fig. 1, chitosan and oligochitosan at 0.5 or 5 g L^{-1} significantly decreased disease incidence. Moreover, at the concentration of 0.5 g L^{-1}, chitosan exhibited better control effect than that of oligochitosan. When the concentration increased to 5 g L^{-1}, there was no significant difference in the control effects of the two antifungal substances.

3.2. Effects of chitosan and oligochitosan on fruit quality

The quality parameters were determined after 3 days of storage (Table 2). As compared to time zero (0 day), fruit firmness, TSS and TA markedly decreased with the storage time. Among them, fruit firmness decreased dramatically. Nevertheless, oligochitosan and chitosan treatments significantly enhanced firmness as compared to control, and the effect of oligochitosan was clearer. The effects of oligochitosan and chitosan at 5 g L^{-1} were more evident than those at 0.5 g L^{-1}. It suggested that the treatments of the two antifungal substances could delay fruit softening and senescence.

3.3. Effects of chitosan and oligochitosan on POD and CAT activities of peach fruit

During the storage at 25 °C, POD activity in peach fruit gradually increased (Fig. 2 A). The treatments of chitosan and oligochitosan at 0.5 or 5 g L^{-1} enhanced the activity as compared to control fruit. Among the treatments, chitosan at 5 g L^{-1} conferred the most evident effect. POD activity in the fruit treated with 5 g L^{-1} chitosan reached the peak at 24 h, and it was almost 3-fold as that in control fruit.

The change pattern of CAT activity in peach fruit stored at 25 °C was shown in Fig. 2B. At 6 h, all the treatments except 0.5 g L^{-1} chitosan enhanced CAT activity. However, fruits in treatment groups showed lower CAT activities than that in control group after 6 h.

3.4. Effects of chitosan and oligochitosan on CHI and GLU activities of peach fruit

The activities of GLU and CHI in peach fruit stored at 25 °C were shown in Fig. 3. All the four treatments enhanced activity of the two enzymes. For GLU activity, 5 g L^{-1} chitosan and 0.5 g L^{-1} oligochitosan showed the most evident inducible effect. At 24 and 48 h, GLU activity reached maximum, and then decreased (Fig. 3B). The activity of CHI in 5 g L^{-1} chitosan treated fruit reached peak at 24 h (Fig. 3A).

3.5. Effects of chitosan and oligochitosan on gene expression of GLU and POD of peach fruit

As shown in Fig. 4, *GLU* and *POD* genes were cloned from peach fruit by RT-PCR, and designated as *GLU* and *POD*. The transcript expression levels of the two genes showed similar change tendency in fruits treated with chitosan or oligochitosan at 5 g L^{-1}. At time 0, the expression level of the two genes was low, especially *GLU*. From 0 to 48 h, *POD* expression increased in control fruit, and reached the

Table 2
Effect of chitosan and oligochitosan with different concentrations on quality of peach fruit stored at 25 °C after 3 days.

	Treatment	Firmness(N)	TSS	TA (H^+ mmol/kg)
0 h		66.6 ± 3.5	9.8 ± 0.5	41.6 ± 1.7
72 h	Control	8.5 ± 1.1	9.3 ± 0.3	40.5 ± 1.5
	0.5 g L^{-1} OCS	13.7 ± 1.1	8.4 ± 0.5	34.9 ± 5.9
	5 g L^{-1} OCS	26.4 ± 1.6	8.2 ± 0.3	33.0 ± 3.4
	0.5 g L^{-1} CS	18.5 ± 1.7	8.3 ± 0.7	31.1 ± 5.7
	5 g L^{-1} CS	24.2 ± 5.7	8.5 ± 0.1	28.5 ± 3.6

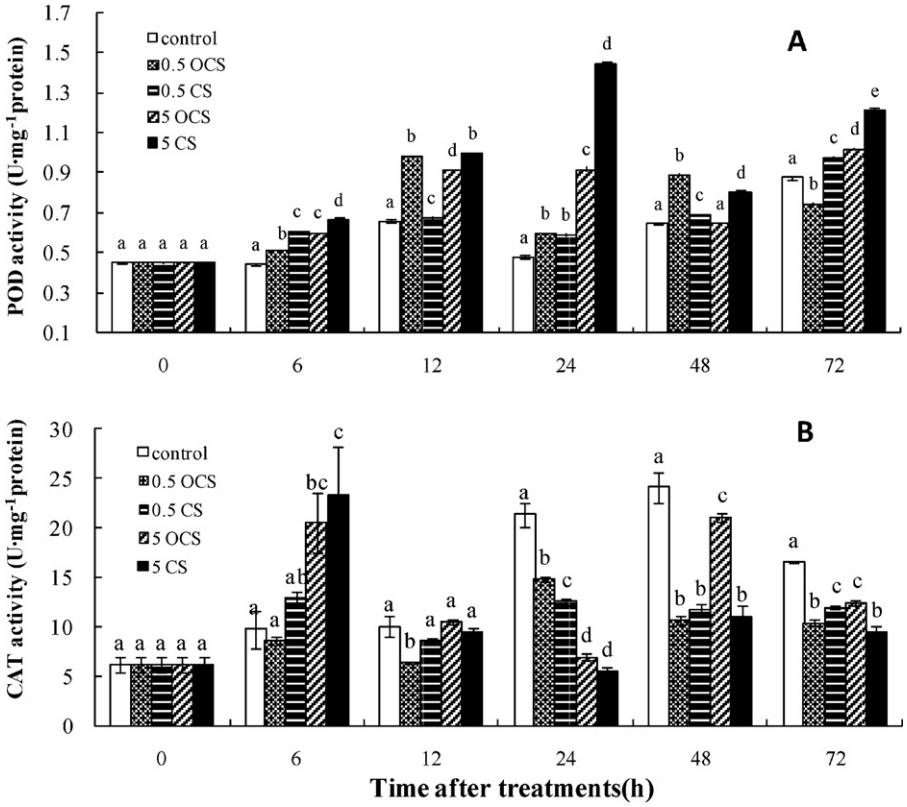

Fig. 2. The activity of peroxidase (POD) and catalase (CAT) in peach fruit treated by chitosan and oligochitoan at different concentrations. Bars represent standard deviations of the means according to three independent experiments. 0.5 g L^{-1} OCS (▩), 0.5 g L^{-1} CS (▤), 5 g L^{-1} OCS (▨), 5 g L^{-1} OCS (■).

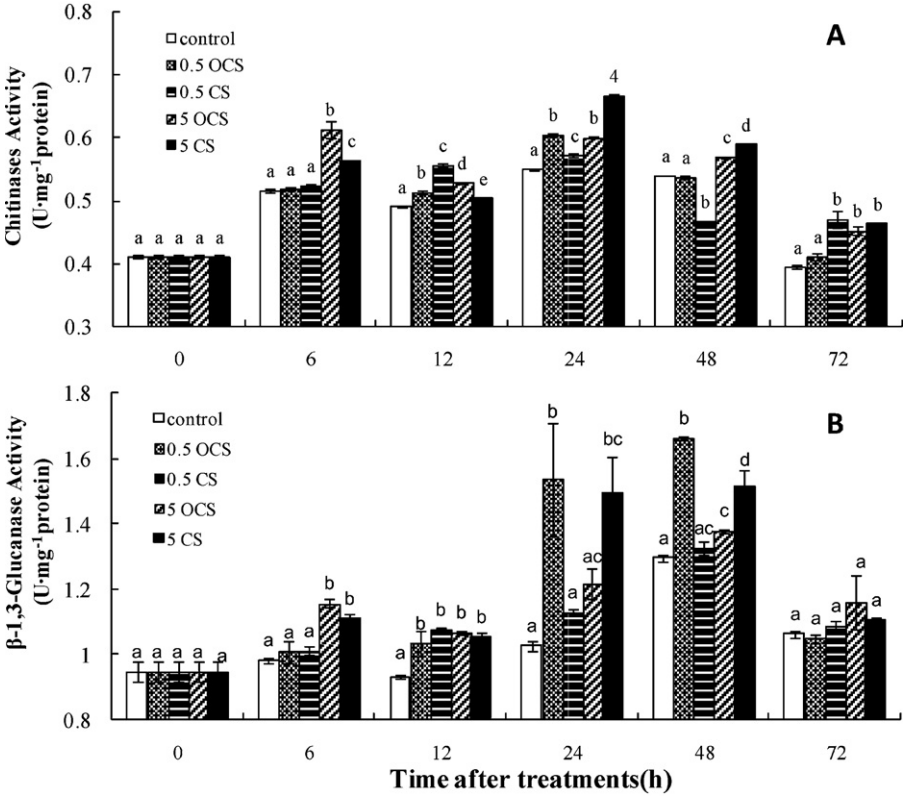

Fig. 3. The activity of β-1,3-glucanase (GLU) and chitinase (CHI) in peach fruit treated by chitosan and oligochitosan at different concentrations. Bars represent standard deviations of the means according to three independent experiments. 0.5 g L^{-1} OCS (▩), 0.5 g L^{-1} CS (▤), 5 g L^{-1} OCS (▨), 5 g L^{-1} OCS (■).

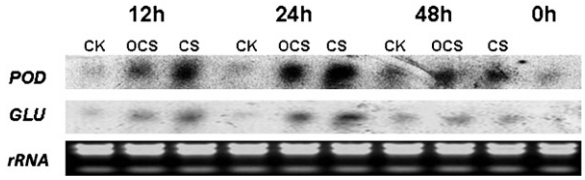

Fig. 4. Effects of chitosan or oligochitosan treatment on the gene expression of *POD* and *GLU* in peach fruit.

maximum at 48 h. However, *POD* expression in chitosan or oligochitosan treated fruit peaked at 24 h, and then decreased at 48 h. Moreover, *POD* expression in treated fruit maintained relatively higher than that in control fruit, and *POD* expression in chitosan treated fruit was the highest. The change in expression level of *GLU* exhibited similar tendency as *POD*, and *GLU* expression level kept relatively lower as compared to *POD*.

4. Discussion

Chitosan and its derivatives have antifungal activity and defense resistance effect. They can trigger defensive mechanisms in plants against pathogenic attacks at very low concentrations (Bautista-Baños et al., 2006). In the present study, we demonstrated that by dipping treatment, chitosan and oligochitosan were effective in controlling brown rot caused by artificial inoculation of *M. fructicola* in peach fruit, and the control effect increased with the treatment concentrations from 0.5 or 5 g L^{-1} (Fig. 1). Similar results were found in other fungal pathogens in previous studies. Bautista-Baños, Hernández-López, Bosquez-Molina, & Wilson (2003) reported that chitosan dipping treatment could inhibit the development of *Colletotrichum gloeosporioides* on papaya, and chitosan at 15 g L^{-1} showed better control effect than that at 5 g L^{-1}. Romanazzi, Nigro, & Ippolito (2003) found that dipping treatment of chitosan reduced storage decay of sweet cherry. These results suggested that chitosan has a protective effect on fruit against postharvest diseases. Although application of oligochitosan on fruit has seldom been reported, it has been found that oligochitosan (DP 3-9) was effective in induction of antiviral resistance and stimulatory effect in tobacco (Zhao, She, Du, & Liang, 2007).

In addition of disease control, the basic quality parameters of peach fruit were also assayed. It was found that chitosan and oligochitosan treatments enhanced firmness and TA, while decreased TSS of peach fruit during storage (Table 2). The results indicated that these treatments could delay ripening and senescence of peach fruit. Such findings were similar to the previous reports obtained in litchi (Ducamp-Collin, Ramarson, Lebrun, Self, & Reynes, 2008). Based on the effects of chitosan and oligochitosan described above, we further investigated the activity of antioxidant and defense-related enzymes in fruit, in order to unveil the related mechanism at the biochemical and physiological level. It was found that the two antifungal substances significantly increased POD and CAT activities in general (Fig. 2). Such results suggested chitosan and oligochitosan exhibited antioxidant capability for peach fruit, as enhancement of POD and CAT enzyme is helpful to eliminate free radicals (Chen, 2008). Oxidative damage caused by ROS contributes to peach fruit senescence (Qin, Meng, Wang, & Tian, 2009). Thus, it was speculated that chitosan and oligochitosan may delay repining and senescence of peach fruit by regulating antioxidant enzyme.

Additionally, we also found that the activity of GLU and CHI increased markedly in chitosan- or oligochitosan-treated peach fruit (Fig. 3). Chitin, as an essential component of the cell wall of many fungal pathogens, can be degraded by CHI. GLU is one of the most fully characterized pathogenesis-related (PR) proteins, and it can act directly by degrading cell walls of pathogens or indirectly by releasing oligosaccharide and eliciting defense reactions. Both of these processes are potential defense mechanism against fungal infection (Tian et al., 2007). The inductive effect of chitosan and oligochitosan on antioxidant and defense response of peach fruit could contribute to the fruit resistance against *M. fructicola* (Fig. 1). In the present study, chitosan and oligochitosan induced the expression of *GLU* as an inducible gene and POD as a constitutive gene, and the inductive effect was most evident at 24 h after treatment (Fig. 4). It corresponded well to the change trend of enzyme activity.

In conclusion, the present study shows that chitosan and oligochitosan, as natural substances, could delay ripening and senescence and effectively control brown rot in peach fruit. They could induct related antioxidant and defense response of peach fruit at enzymatic and transcript level. This suggests that they are promising to partially substitute the utilization of synthetic fungicides for postharvest diseases, but further study on the large-scale application of chitosan and oligochitosan is still needed.

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