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Full Length Research Paper

Defense responses in tomato fruit induced by oligandrin against *Botrytis cinerea*

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Oligandrin is known to induce resistance against a number of plant diseases. However, its effects on postharvest diseases are still unclear. The effects of oligandrin on the control of postharvest diseases in tomato fruit and its underlying mechanisms were investigated in this study. The treat01ent of tomato fruit with oligandrin (10 µg/ml) significantly reduced the incidence and severity of gray mould (caused by *Botrytis cinerea*). After 5, 7 and 9 days of artificial inoculation, the relative cure effect was 60.5, 52.1 and 48.5%, respectively. The results from bio-assay indicated that the treatment stimulated the activity of the defense related enzymes. Phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD) activity in the oligandrin-treated fruit was about 39.2, 69.6 and 52.6% higher than that in control on the 3rd day, respectively. Furthermore, mRNA level of the genes encoding pathogenesis-related proteins (PRs), such as *PR-2a* (extracellular β-1, 3 -glucanase) and *PR-3a* (extracellular chitinase) in tomato fruit was about 2.7-fold and 4.6-fold above that of the control at the peak stage. The expression of LeERF2 and PR6, which confer an ethylene-dependent signaling pathway, were also significantly increased 6.6-fold and 3.6-fold by such treatment. These results indicate that oligandrin has the potential to control gray mould and it may play an important role in the induction of resistance to *B. cinerea* and the activation of the ethylene-dependent signaling pathway.

Key words: Tomato, disease resistance, oligandrin, *Botrytis cinerea.*

INTRODUCTION

Fungal plant pathogens are known to cause considerable postharvest loss of fruit and vegetables (Tripathi and Dubey, 2004). Tomato (*Solanum lycopersicum*) fruit usually have a very short postharvest life. Decay is an important factor, which limits the storage life of tomato, and results in appreciable losses at wholesale, retail, and consumer levels. Gray mould decay caused by *Botrytis cinerea* is one of the most important postharvest diseases of tomato fruit (Liu et al., 2005). Although post-harvest diseases can be controlled by synthetic fungicides (Eckert and Ogawa, 1988), the use of fungicides has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and possible side-effects on human health through the food (Tripathi and Dubey, 2004). For this reason, new alternatives have been explored all over the world to reduce the use of synthetic fungicides.

The natural resistance of fruit and vegetables to disease usually declines after harvesting, leading to infection by pathogens. Elicitors, as a part of integrated pest management (IPM) approach, are usually used to induce resistance against postharvest diseases (Terry and Joyce, 2004). Today a number of elicitors enhancing disease resistance in postharvest horticultural crops have been studied, including calcium chloride, chitosan, salicylic acid (SA), oxalic acid, and the antagonistic yeast *Cryptococcus laurentii* (Molloy et al., 2004; Tian et al., 2006; Liu et al., 2007). It has been proven that induced resistance as an alternative for the control of postharvest diseases in fruit is effective in both the laboratory and a few cases in the field (Tian and Chan, 2004).

Oligandrin, secreted from *Pythium oligandrum* was found to share some similarities with several elicitins from other *Phytophthora* spp. and *Pythium* spp. However, oligandrin did not induce hypersensitive reactions. Treatment with oligandrin conferred enhanced resistance against *Phytophthora parasitica* and *Fusarium oxysporum*

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f. sp. *radicis-lycopersici* in tomato plant (Benhamoua et al., 2001; Lherminier et al., 2003). Oligandrin-treated plants react more rapidly and more efficiently to *F. oxysporum*. f. sp. *radicis-lycopersici* attack, mainly through the massive accumulation of mycotoxic compounds at sites of attempted pathogen penetration (Lou et al., 2005; Picard et al., 2000). The gene expression and activity of pathogenesis-related (PR) proteins such as chitinases and β-1, 3-glucanase also have been largely studied and related to defense response (Bezier et al., 2002; Robert et al., 2002).

However, little is known on the defense response against postharvest diseases and the underlying mechanisms in fruit treated with oligandrin. Here, we demonstrated the potential of oligandrin and its possible mechanisms in controlling gray mould in tomato fruit.

MATERIALS AND METHODS

The protein of oligandrin

Oligandrin (10 μg/ml) was provided by the laboratory used for this study (Institute of Biotechnology, Zhejiang University, Hangzhou, China).

Fruit material and the oligandrin treatment

Tomato (*S. lycopersicum* Hezuo903) fruit were harvested from commercial orchard at the mature red stage (surface color of the fruit was red) and selected for uniformity of size, ripeness and absence of defects. Tomato fruit were washed with running tap water, surface sterilized with 2% sodium hypochlorite for 3 min, rinsed twice in sterile distilled water and dried in ambient air. These fruit were then uniformly treated with water or oligandrin (10 μg/ml) 24 h before inoculation with *B. cinerea*, all containing 0.03% (v/v) Tween-80 on their surface and immediately transferred to an air-tight plastic bag to maintain a high relative humidity and incubated at 25°C. The effects of oligandrin as a control of postharvest diseases in tomato fruit were examined.

Pathogen inoculums

B. cinerea fq01 was isolated from decayed tomato fruits and maintained on potato dextrose agar (PDA). Conidial suspensions of the pathogen were obtained from 2-week-old cultures incubated at 25°C by flooding the cultures with sterile distilled water. The concentration of spores was adjusted to 1.0×10^5 spores ml^{-1} with a hemocytometer prior to use.

Effects of treatment with oligandrin on postharvest disease of tomato fruit

The experiment was conducted under artificial inoculation. At 24 h after oligandrin or sterile distilled water treatment, tomato fruit were wounded 4 mm deep with a toothpick at the equator. Then 5 μl of the conidial suspension of *B. cinerea* at 1.0×10^5 spores ml^{-1} was added to each wound, and water was used as the control. Inoculated fruits were put in 380 × 250 × 80 mm plastic boxes, and then put in artificial climate incubator in order to keep them at 25°Cwith 85 to 95% RH. Disease incidence and disease index caused by *B. cinerea* were determined after inoculation for five days. Each treatment contained three replicates with 20 fruit per replicate.

A scale of 0 to 4 was used to describe disease severity. The degree of disease severity inoculation with *B. cinerea* comprised 0: no lesion; 1: lesion diameter <10 mm; 2: lesion diameter 10 to 20 mm; 3: lesion diameter 20 to 30 mm; and 4: lesion diameter >30 mm. The disease incidence and disease index for each treatment were calculated using the following formula:

$$\text{Disease index} = (\sum_{i=0}^{4} N_i \times =) / (4 \times \sum_{i=0}^{4} N_i)$$

Where, i is the severity (0 to 4), 4 is the highest level, and N_i are the number of fruit with a severity of i.

Enzyme activity assay of PAL, PPO, POD and phenolic compounds in tomato fruit

For enzyme assays, fresh fruit tissue samples 1 to 2 mm under the surface were collected at different times after treatment. All enzyme extract procedures were conducted at 4°C. For PAL, 1 g of the tissue was ground with 2 ml extracting buffer [0.2 M boric acid buffer containing 10% (w/v) polyvinypoly-pyrolidone (PVPP), 1 mM EDTA, and 50 mM β-mercaptoethanol, pH 8.8]. For PPO and POD, 2 g of the tissue were ground with 10 ml of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of PVPP. The extracts were then homogenized and centrifuged at 12,000 × g at 4°C for 30 min, and the supernatant was collected.

For the PAL assay, 300 μl of the extract was incubated with 1 ml 0.02 M L-phenylalanine and 2 ml of the PAL extracting buffer at 24°C for 2 min, and absorbance at 290 nm was measured in an ultraviolet spectrophotometer. The PAL activity was assayed as U_{290}, where $U_{290} = 0.01 \triangle OD_{290}$/mg protein/min (Assis et al., 2001).

For the PPO assay, 100 μl of extract was incubated with 2 ml 0.05 M phosphate buffer (pH 7.0) and 0.5 ml 0.5 M catechol at 24°C for 2 min, and absorbance at 398 nm was measured with an ultraviolet spectrophotometer. The PPO activity was expressed as U_{398}, where $U_{398} = 0.01 \triangle OD_{398}$/mg protein/min (Galeazzi et al., 1981).

POD activity was determined using guaiacol as the substrate (Ippolito et al., 2000). The reaction mixture consisted of 0.1 ml of crude extract and 2 ml of guaiacol (8 mM, in 100 mM sodium phosphate buffer, pH 6.4), incubated for 30 min at 30°C. The increase in absorbance at 460 nm was measured after 1 ml H_2O_2 (24 mM) was added. The activity of POD was expressed as U_{460}, where $U_{460} = 0.01 \triangle OD_{460}$/mg protein/min.

Protein content was determined using Nanodrop ND-1000 Spectrophotometer at OD_{280}.

RNA isolation

At different times after oligandrin and water treatment, fresh tomato fruit samples of 1 to 2 mm under the surface were detached and stored at −80°C. Total RNA was extracted using RNA isolation kit (Invitrogen, USA). The isolated RNA was dissolved in 20 μl of Rnase free water, quantified by spectrophotometry and stored at −80°C.

cDNA clones

2 μg of total RNA extracted from tomato fruit were reverse transcribed with reverse cDNA kit (Invitrogen) and stored at −80°C.

Real-time PCR

The reactions (20 μl) contained Bio-Rad Super SYBR Green mix

(10 µl), 20 ng cDNA (1.0 µl) each primer (10 µM) (0.2 µl), and ddH_2O (8.6 µl). The PCR reactions were dispensed into ABI optical reaction tubes (Applied Biosystems, Foster City, CA, USA). The reaction tubes were centrifuged at 3,000 rpm for 10 s to settle the reaction mixtures to the bottom of the wells. The PCR was carried out with an icycler real-time quantity PCR system (BIO-RAD). The thermal profile consisted of an initial hold for 94°C for 3 min, 1 cycle, 95°C for 45 s, 52°C for 45 s, 72°C for 60 s, 35 cycles and 72°C for 10 min. After each run, a dissociation curve was designed to confirm specificity of the product and avoid production of primers-dimers.

The gene for β-actin was used as a housekeeping gene. Calculation of relative quantification was done by the comparative $2^{-\Delta\Delta Ct}$ methods (Livak and Schmittgen, 2001). All reactions were performed in triplicate. The name and sequence of the primer as (5'-3'):

β-actin: R-5'- GAGTTGACCTGCCATTT -3', F-5'-TTTCACGATTAGCCTTT- 3'.
PR-2a: R-5'- TTGGGATAAACTATGGACG-3', F-5'-TGGATGGGTAATAAGCAG -3'.
PR-3a: R 5'- GTCAAACATCTCACGAAACT -3', F 5'-TGTCGCAACTAAATCAGG -3'
ERF2: R 5'- ACGACTTCCTTGCTGTGA -3', F 5'-TCTGGTCCTATCCATTTCC -3'
PR6: R 5'-TCCTTGCTCACCTACTTGTTCTTGG -3',
F5'-TTCCTTATGCTGTGGAAATACTTTG -3'

Statistical analysis

All statistical analyses were performed with statistical program from social sciences (SPSS) 10.0. Data were analyses by one-way ANOVA. Mean separations were performed by Duncan's multiple range tests. Differences at $P< 0.05$ were considered to be significant.

RESULTS

Effect of oligandrin treatment on postharvest disease of tomato fruit

In this experiment, the treatment with oligandrin (10 µg/ml) for 24 h before inoculation with *B. cinerea* had significantly protective and curative effect against gray mould in the tomato fruits. Tomato fruits were naturally infected by several pathogens, such as *B. cinerea*, *Septoria lycopersici* and *Alternaria alternate*. Since the gray mould caused by *B. cinerea* was the main natural disease, we further studied the effect of oligandrin on the control of gray mould inoculation with *B. cinerea*. As shown in Figure 1, the combination of 10 µg/ml oligandrin significantly inhibited the gray mould inoculation with *B. cinerea*.

Treatment with the oligandrin significantly reduced the disease index and disease incidence in tomato fruit inoculation with *B. cinerea* in 15 days after treatment ($P< 0.05$, Figure 1). After inoculation for 5, 7 and 9 days, the relative cure effect was 60.5, 52.1 and 48.5%, respectively. It was suggested that the oligandrin had significant protective effect against gray mould caused by *B. cinerea* in tomato fruit.

Effect of oligandrin treatment on activities of PAL, PPO and POD in tomato fruit

Treatment of the oligandrin increased the activities of PAL, PPO and POD in tomato fruit when stored at 25°C. PAL, PPO and POD activities in the oligandrin-treated fruit were about 39.2, 69.6 and 52.6% higher than that in the control on the 3rd day after treatment, respectively (Figure 2).

In the fruit inoculated with *B. cinerea*, PAL, PPO and POD activities reached the highest values at the 3rd day in the oligandrin-treated fruit, and the level were almost 18.8, 20.0 and 22.7% higher than that in the inoculated fruit at the same time, respectively (Figure 2).

Oligabdrin induces the expression of defense-related genes

Oligandrin treatments enhanced the expression of PR-2a (extracellular β-1, 3-glucanase) and PR-3a (extracellular chitinase) genes (Figure 3). Transcription of the PR-2a gene reached its maximum level at 24 h in oligandrin treated tomato fruit, and the level was about 2.7-fold higher than that in control fruit at the same time point (Figure 3A). After that, the expression rapidly declined. The control tomato fruit showed a continuous lower mRNA level of *PR-2a* throughout the experiment.

The changes in PR-3a mRNA levels showed a similar pattern to that of PR-2a. PR-3a mRNA levels in oligandrin treated tomato fruit were about 4.6-fold above that in the control fruit at the peak stage (Figure 3B).

The expression of both PR6 and LeERF2 genes were transiently induced at 8 and 24 h in oligandrin-treated fruits (Figure 4). The expression of PR6 in oligandrin treated tomato fruit were about 3.6-fold and 1.0-fold higher than that in control fruit at 8 and 24 h. The changes in LeERF2 mRNA levels showed a similar pattern to that of PR6. LeERF2 mRNA levels in oligandrin treated tomato fruit were about 6.6-fold and 7.4-fold above that in the control fruit at 8 and 24 h. Increased production of LeERF2 and PR6 resulting from oligandrin treatments in tomato fruits suggested that an ethylene-dependent signaling pathway might be activated in tomato fruits.

DISCUSSION

As an elicitor, oligandrin can induce resistance against multi-diseases in plants (Benhamou et al., 1999; Lou and Zhang, 2005; Hase et al., 2006; Wang et al., 2007). Our results revealed that application of oligandrin significantly increased the transcript level of PR-2a and PR-3a (Figure 3). It also evoked the activities of PAL, PPO and POD and effectively inhibited gray mould in tomato fruit (Figure 1). These results are consistent with the previous findings that oigandrin enhances disease resistance in cucumber

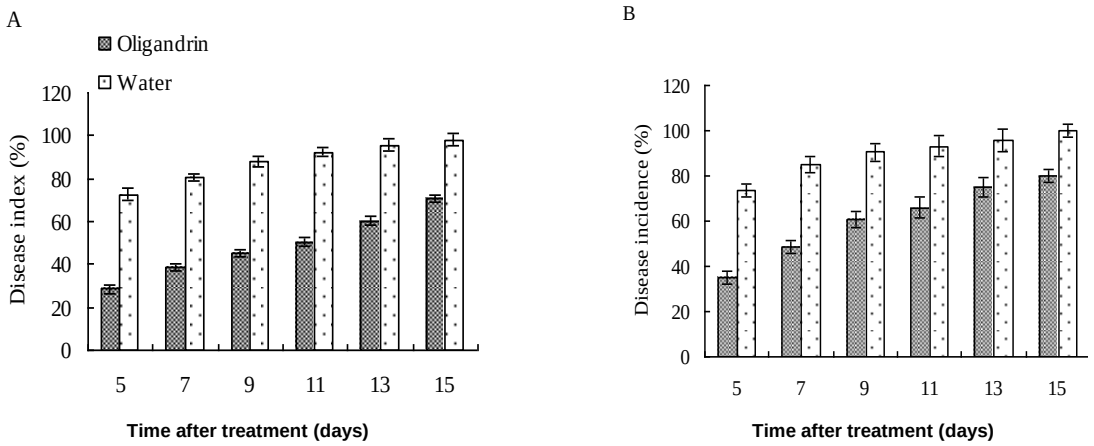

Figure 1. Effects of oligandrin on disease caused by *B. cinerea* in tomato fruit at 25°C. Bars represent standard deviations of the means.

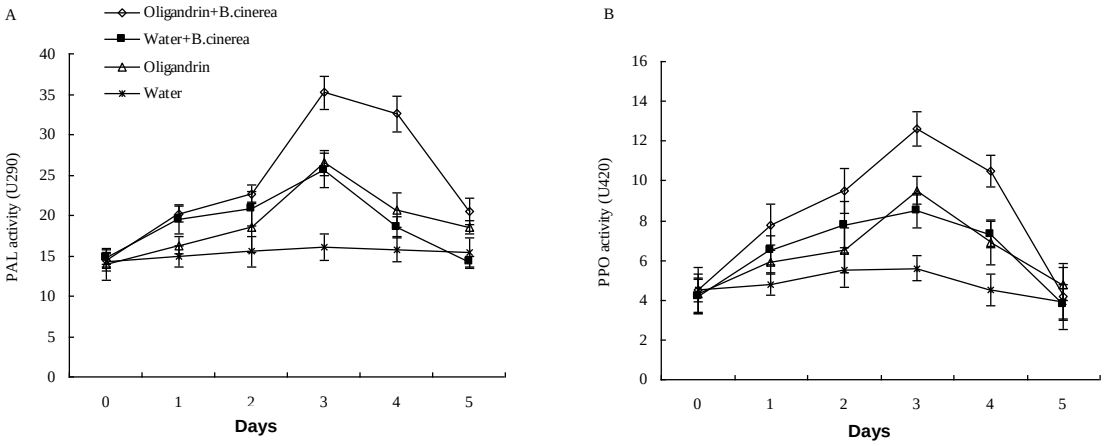

Figure 2. Changes in PAL (A), PPO (B) and POD (C) activities in tomato fruit. Tomato fruit were treated with water, oligandrin or oligandrin and water for 24 h before inoculated with *B. cinerea* (1.0×10^5 spores per ml). Afterwards, the fruit were incubated at 25°C, 85 to 95% RH. Bars represent standard deviations of the means.

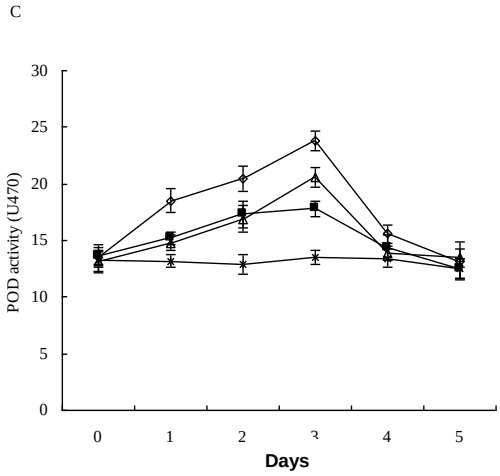

Figure 2. Contd.

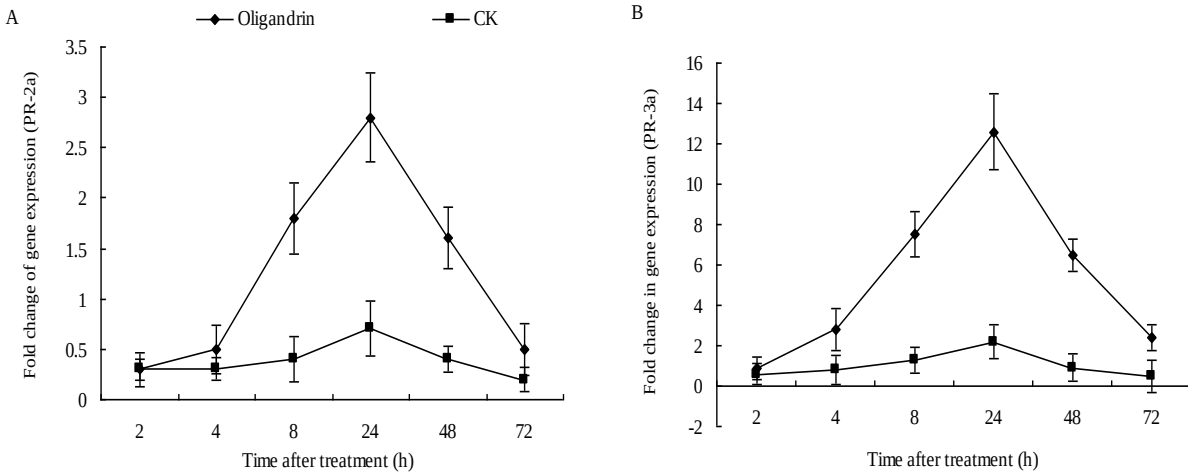

Figure 3. Induction of defense gene. A, *PR-2a* (extracellular β-1, 3-glucanase); B, *PR-3a* (extracellular chitinase) expression by oligandrin in tomato fruit at 2, 4, 8, 24, 48 and 72 h (method of $2^{-\Delta\Delta Ct}$). Bars represent standard deviations of the means.

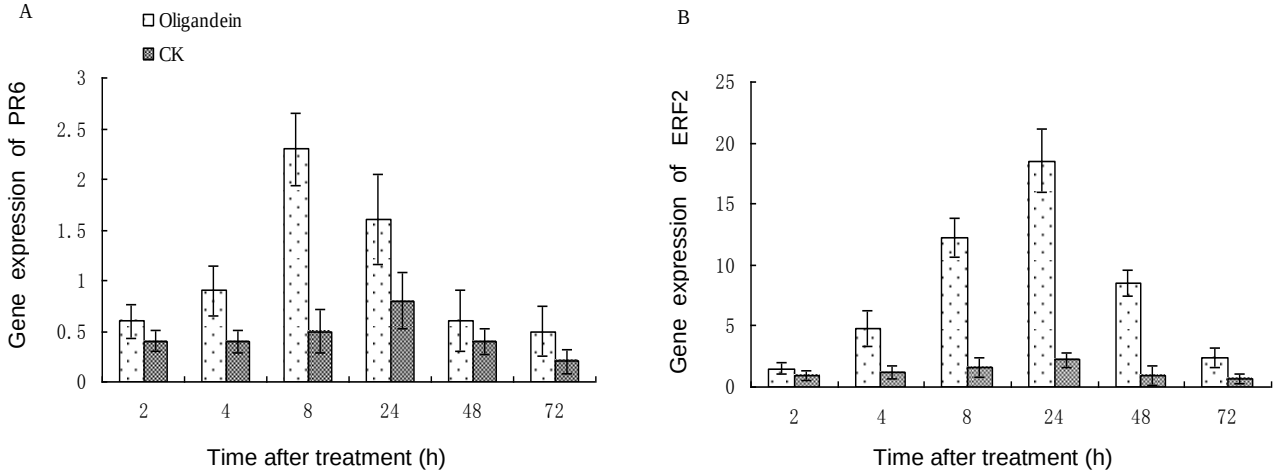

Figure 4. The defense gene (A is PR6 and B is ERF2) expression in tomato fruits induced by oligandrin at 2, 4, 8, 24, 48 and 72 h (method of $2^{-\Delta\Delta Ct}$).

and tomato seedlings (Picard et al., 2000).

The expression of PR genes has been taken as markers of the systemic acquired resistance (SAR) (Ward et al., 1991). In this study, the induced transcription of PRs by oligandrin suggested that the mechanism of increasing postharvest disease resistance may be related to SAR in tomato fruit. PRs can be induced by different stress stimuli and play important roles in plant defense against pathogenic constraints and general adaptation to stressful environments (Edreva, 2005). The family of PR-2 catalyze endo-type hydrolytic cleavage of the 1,3-β-d-glucosidic linkages in β-1,3-glucans. PR-3a is endo-chitinases that catalyze the hydrolysis of β-1,4-N-acetylglucosamine linkages, so they can cleave fungal cell walls *in situ* and play a major role in disease resistance. Transgenic tobacco over expressing PR-2 and PR-3 has been shown to have improved resistance to *Cercospora nicotianae* (Zhu et al., 1994). Combining chitinases with β-1,3-glucanases can significantly enhance biocontrol efficacy against many pathogenic fungi (Sela-Buurlage et al., 1993). In this study, oligandrin increased mRNA levels of the acidic PRs (PR-2a and PR-3a) (Figure 3), which might effectively inhibit fungal infection and restrict expansion of the pathogen.

In general, the activation of the ethylene-dependent signaling pathway is accompanied by the expression of genes encoding ethylene receptors and/or ethylene-responsive transcription factors (Gutterson and Reuber, 2004; Hase et al., 2006). Transcription of ethylene receptor homologue PR6 and ethylene-inducible transcription factor LeERF2 in the fruits were analysed and the expression of both PR6 and LeERF2 genes were

transiently induced at 8 and 24 h in oligandrin-treated fruits (Figure 4). These results indicate that the transient increase of ethylene production was likely to have been associated with the activation of the ethylene mediated signaling pathway and induction of basic PR gene expression in mycelial homogenate and oligandrin-treated tomato fruits, which resulted in the reduction of gray mould disease caused by *B. cinerea*. Defense responses in tomato fruit against *B. cinerea* induced by oligandrin had the features of both induced systematic resistance (ISR) and systematic acquired resistance (SAR). Oligandrin perhaps induced salicylic acid (SA), jasmonic acid and ethylene (JA/ET) signal pathway together.

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REFERENCES

Assis JS, Maldonado R, Munoz T, Escribano MI, Merodio C (2001). Effect of high carbon dioxide concentration on PAL activity and phenolic contents in ripening cherimoya fruit. Postharvest Biol. Technol. 23: 33-39.

Benhamou N, Rey P, Picard K, Tirilly Y (1999). Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soilborne plant pathogens. Phytopathology, 89: 506-517.

Benhamoua N, Bélangera RR, Rey P, Tirilly Y (2001). Oligandrin, the elicitin-like protein produced by the mycoparasite *Pythium oligandrum*, induces systemic resistance to Fusarium crown and root rot in tomato plants. Plant Physiol. Biochem. 39: 681-698.

Bezier A, Lambert B, Baillieual F (2002). Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. Eur. J. Plant Pathol. 108: 111-120.

Eckert JW, Ogawa JM (1988). The chemical control of postharvest diseases: deciduous fruits, berries, vegetables and root/tuber crops. Annu. Rev. Phytopathol. 26: 433-469.

Edreva A (2005). Pathogenesis-related proteins: research progress in the last 15 years. Gen. Appl. Plant Physiol. 31: 105-124.

Galeazzi MAM, Sgarbieri N, Costantinides SM (1981). Isolation, purification and physiochemical characterization of polyphenol oxidase from dwarf variety of banana (*Musa Cavendishii*). J. Food Sci. 46: 150-155.

Gutterson N, Reuber TL (2004). Regulation of disease resistance pathways by AP2/ERF transcription factors. Current Opinion in Plant Biology 7: 465–471.

Hase S, Shimizu A, Nakaho K, Takenaka S, Takahashi H (2006). Induction of transient ethylene and reduction in severity of tomato bacterial wilt by *Pythium oligandrum*. Plant Pathol. 55: 537-543.

Ippolito A, Elghaouth A, Wilson CL, Wisniewski M (2000). Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biol. Technol. 19: 265-272.

Liu HX, Jiang WB, Bi Y, Lou YB (2005). Postharvest BTH treatment induces resistance of peach (*Prunus persica* L. cv. Jiubao) fruit to infection by *Penicillium expansum* and enhances activity of fruit defense mechanisms. Postharvest Biol. Technol. 35: 263-269.

Liu J, Tian SP, Meng XH, Xu Y (2007). Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. Postharvest Biol. Technol. 44: 300-306.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402-408.

Lou BG, Zhang BX (2005). Biocontrol and induction of defenses by the non-pathogenic *Pythium* spp. Acta Phytophylacica Sinica 32(1): 93-96.

Molloy C, Cheah LH, Koolaard JP (2004). Induced resistance against *Sclerotinia sclerotiorum* in carrots treated with enzymatically hydrolysed chitosan. Postharvest Biol. Technol. 33: 61–65.

Picard K, Ponchet M, Blein J, Rey P, Tirilly Y, Benhamou N (2000). Oligandrin, a proteinaceous molecule produced by the mycoparasite *Pythium oligandrum* induces resistance to *Phytophthora parasitica* infection in tomato plants. Plant Physiol. 124: 379-395.

Robert N, Roche K, Lebeau Y, Breda C, Boulay M, Esnault R, Buffard D (2002). Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. Plant Sci. 162: 389-400.

Sela-Buurlage MB, Ponstein AS, Bre-Vloemans SA, Melchers LS, van den Elzen PJM, Cornelissen BJC (1993). Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol. 101: 857-863.

Terry LA, Joyce DC (2004). Elicitors of induced disease resistance in harvested horticultural crops: a brief review. Postharvest Biol. Technol. 32: 1-13.

Tian SP, Chan ZL (2004). Potential of induced resistance in postharvest diseases control of fruits and vegetables. Acta Phytopathol. Sinica 34: 385-394.

Tian SP, Wan YK, Qin GZ, Xu Y (2006). Induction of defense responses against *Alternaria* rot by different elicitors in harvested pear fruit. Appl. Microbiol. Biotechnol. 70:729-734.

Tripathi P, Dubey NK (2004). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. Postharvest Biol. Technol. 32: 235-245.

Wang AY, Lou BG, Xu T (2007). Inhibitory effect of the secretion of *Pythium oligandrum* on plant pathogenic fungi and the control effect against tomato gray mould. Acta Phytophylacica Sinica 34(1): 57-60.

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Metraux JP, Ryals JA (1991). Coordinate gene activity in response to agents those induce systemic acquired resistance. Plant Cell 3: 1085-1094.

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994). Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Nature Biotechnol. 12: 807-812.