Defense response of tomato fruit at different maturity stages to salicylic acid and ethephon

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In order to elucidate whether fruit maturity stage influence the induced resistance of exogenous elicitors in tomato and the involved mechanisms, we investigated the defense responses of tomato fruits against Botrytis cinerea, ethylene production and internal quality following treatments of fruit with salicylic acid (SA) or ethephon (ET) at mature green (MG) and breaker (BR). SA significantly suppressed decay and disease incidence in tomato fruits at both MG and BR stages, along with higher expression level of PR1 gene after 2 days of treatment. All fruits treated by SA had lower contents of ethylene and lycopene. The ET-treated fruit at both maturity stages showed lower disease incidence and higher level of PR2 and PR3 expression compared with the control fruit. ET treatment significantly enhanced ethylene and lycopene contents, and accelerated fruit ripening. Our results suggest that SA and ET induced disease resistance in fruits by mediating the expression of different pathogenesis-related genes and have different effects on fruit ripening, which in turn influences the disease resistance of tomato fruits.

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1. Introduction

Fungal diseases during fruit storage cause the huge economic losses. Among many strategies for control of postharvest diseases in fruit, activation of inducible defense systems was particularly attractive (Terry and Joyce, 2004; Tian et al., 2006). In recent years many elicitors, such as salicylic acid, silicon, brassinosteroids and oxalic acid, have been proved to effectively induce fruit resistance to fungal pathogens (Zeng et al., 2006; Qin and Tian, 2005; Zhu et al., 2010; Wang et al., 2009). However, limited information such as the time to apply elicitors restrains their practical application. A better understanding of the mechanisms through which the induced resistance is activated in fruit at different maturities is essential for the use of elicitors in controlling fungal diseases during fruit storage.

Unlike plant vegetative tissue, fruit undergoes a mature and ripening process, resulting in great biochemical and physiologically changes, including sugar/acid ratio and fruit softening. Such changes consequently provide nutrients for pathogens and activate the fungal pathogenicity factors (Prusky, 1996). In climacteric fruit, these processes are often coupled with the evolution of ethylene hormone, which induces expression of the ripening-related genes and increases susceptibility of fruit to pathogen (Giovannoni, 2001). Apart from its role in fruit ripening, ethylene also activates the expression of plant defense-related proteins which might be associated with fruit resistance to pathogens (van Loon et al., 2006a). The existence of ethylene during fruit ripening increases the complexity of exogenous elicitors in inducing resistance. In tomato, postharvest treatment with different elicitors such as methyl jasmonate (MeJA), ultra-light C (UV-C) and chitosan were confirmed to be effective in controlling decays caused by Botrytis cinerea (Yu et al., 2009; Charles et al., 2009; Badawy and Rabea, 2009). Most of these researches have focused on fruits at green stage of maturity probably because ethylene production at this stage is of low level and ripening qualities is not completely formed. In fact, however, to acquire good qualities in commercial, tomato fruit are usually picked at higher ripening degree than mature-green stage. Therefore, it is important to evaluate the effect of elicitors on fruit defense response at other stages of maturity.

Salicylic acid (SA) plays a central role in plant resistance (Sticher et al., 1997). Exogenous application of SA increases resistance to fungal pathogen in sweet cherry (Yao and Tian, 2005) and grape berries (Derckel et al., 1998). Such an increased resistance was
found to be correlated with enhanced expression or/and activities of glucanase and chitinase (Yao and Tian, 2005; Derckel et al., 1998). 2-Chloroethylphosphonic acid (ethephon; ET) is often used as a substitute for ethylene in triggering the ethylene signaling pathway (van Kan et al., 1995). Treatment with ET induces glucanase and chitinase expression in plant (van Kan et al., 1995). However, the effects of ET or ethylene on disease development are still unclear (Barkai-Golan et al., 2008), and few studies investigate their role in disease resistance during fruit postharvest. In this study, we used SA and ET as exogenous elicitors to treat tomato fruit at different maturity stages. We examined the resistance of tomato fruit against *B. cinerea*; the expressions of PR1, PR2, and PR3; and their effects on ethylene production and fruit qualities to understand whether fruit maturity stage influences the defense response of fruit to SA and ethylene.

2. Materials and methods

2.1. Fruits and treatments

Tomato (*Lycopersicon esculentum* L. cv. Fenhong) fruits were harvested at two maturity stages according to the description by Mitcham et al. (1989): mature green (MG) (fully expanded but unripe fruit with mature seed) and breaker (BR) (first visible sign of carotenoid accumulation on bottom). Fruits were selected based on the uniform size and no physical injuries or infections. Prior to use, fruits were surface-disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water, and air-dried. Then, fruits at each stage were immersed in different solutions (5 mmol L\(^{-1}\) SA, 0.05 mmol L\(^{-1}\) ET, and distilled water) for 15 min. After drying in air, each group of fruits was further divided into two parts. One was for the inoculation experiment; the other was directly placed into plastic boxes with approx. 90% relative humidity (RH), stored at 20 °C, and sampled from fruit pericarp at various time intervals after treatments.

2.2. Pathogen and inoculation

*B. cinerea* was originally isolated from infected tomato fruit showing a typical gray mold symptom. The isolate was purified by a single spore isolation technique and identified based on cultural and morphological characteristics and cultured on potato dextrose agar (PDA) plates at 25 °C for 7 days. Spores were obtained by flooding the surface of the culture with sterile distilled water containing 0.05% Tween-80 (v/v). Spore suspension was filtered through four layers of sterile cheesecloth to remove any hyphal fragments. The suspension concentration was adjusted to 1 × 10^5 spores mL\(^{-1}\) using a hemocytometer.

Tomato fruits were treated with either SA or ET as described above. After treatment for 24 h, fruits were wounded (4 mm depth and 3 mm width) with a sterile nail and inoculated with one drop (10 μL) of the spore suspension on the surface of each wound and stored at 20 °C with 90% RH. Disease incidence and lesion diameter were measured daily according to the following formulas until all control fruits decayed. There were 3 replicates in each treatment with 15 fruits, and the experiment was repeated twice.

Disease incidence (%) = \[\frac{\sum\text{Number of decayed tomato fruit}}{\sum\text{Total number of treated fruit}} \times 100\]

Lesion diameter (mm) = \[\frac{\sum\text{Lesion diameter of decayed tomato fruit}}{\sum\text{Total number of treated fruit}}\]

2.3. RNA isolation and semi-quantitative RT-PCR analysis

RNA isolation and semi-quantitative RT PCR analysis were performed as described previously by Moore et al. (2005) and Spencer and Christensen (1999), respectively. The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) (gi: U93208) was used as the internal control to normalize cDNA templates. PCR conditions used were: 94 °C for 3 min, 33 cycles of 94 °C for 30 s, 57 °C for
Fig. 2. Effects of salicylic acid (SA) and ethephon (ET) treatment on the expression of PR1, PR2 and PR3 in tomato fruit at mature green stage (MG) (A, C, E and G) and breaker stage (BR) (B, D, F and H), treated with 0.5 mmol L\(^{-1}\) ET or 5 mmol L\(^{-1}\) SA or water (control), respectively. Semi-quantitative RT-PCR was conducted using fruits at 1 and 2 days after treatment, as well as 0 days for control. Graphics represent the quantification of the intensity of amplified DNAs and vertical bars the standard deviations. Different letters above bars are significantly different at \(p < 0.05\) according to Duncan’s multiple range tests.

30 s, 72 °C for 1 min, and finally 72 °C for 10 min, with sequences of the primers sense (5′-TCT TTG GWA GAG AAG GCT GTT AC-3′) and antisense (5′-GGR ATR ATG TTG AAY GAA GC-3′). To determine the relative transcript levels of other genes, normalized cDNA were used as the templates and the same PCR conditions were used except the number of PCR cycles, by which a minimal signal density can be shown. Specific primers used for PCR were designed to amplify across intron regions, including PR1 (gi: EU589238) sense (5′-ACA AGT TGG AGT CGG TCC T-3′) and antisense (5′-ACA GTG GAG CGG TCC T-3′) and antisense (5′-TCA CAT AAG CAC TCT GA-3′), PR-2 (gi: M80608) sense (5′-CTG GTT TGG GAA ATG AC-3′) and antisense (5′-AGG AAC ATT CAC TGG AGG AT-3′) and antisense (5′-AAT AAA GAC GCT TAG CCC TG-3′). The intensity of amplified DNA was quantified using Scion Image software (Scion Corporation, Frederick, MD, USA) and the relative expression abundance of each gene was expressed as the ratio of sample intensity to GAPDH intensity. Each treatment contained three replicates and the entire experiment was repeated twice.

2.4. Ethylene production and quality assessment

To measure the ethylene production, fruits were placed into glass containers (6.14 L) and sealed for 1 h at 20 °C. 1 mL gas sample was withdrawn from the headspace volume of the container with a syringe and analyzed using the gas chromatograph (SQ-206, Beijing, China) equipped with a photo ionization detector (PID). The column temperature was set at 80 °C and the oven was at 150 °C. The gas pressure was set at 0.06 MPa for carrier gas (nitrogen), 0.04 MPa for flammable gas (hydrogen), and 0.05 MPa for supporting gas (air). The carrier gas flow rate was 30 mL min\(^{-1}\). Each treatment contained three replicates and the entire experiment was repeated twice.

Pericarp lycopene content was measured after 4 days of storage according to the method of Choi and Huber (2008). Lycopene content was calculated using the molar extinction coefficient of 17.2 L mol\(^{-1}\) m\(^{-1}\), and expressed as mol g\(^{-1}\) fresh weight. Fruit soluble solids content (SSC) and pH value were detected by an Abbe refractometer (10481 S/N, USA) and by a pH analyzer (pH analyzer, CPHS-32B, Shanghai, China), respectively. Each treatment contained three replicates and the entire experiment was repeated twice.

2.5. Statistical analysis

All statistical analyses were performed with SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Data from assay of disease development, gene expression and lycopene content were analyzed by one-way analysis of variance (ANOVA), and the mean separations were performed by Duncan’s multiple range tests. Differences at \(p < 0.05\) were considered as significant.
3. Results

3.1. Disease development in tomato fruits

Application of SA significantly suppressed disease incidence and reduced lesion diameter of *B. cinerea* in fruits at both MG and BR stages compared to the control (*p* < 0.05). The disease incidences in ET-treated fruits at both maturity stages were suppressed after incubation for 3 days at room temperature, but the effect did not last until 4 days after incubation (Fig. 1). Moreover, disease incidence and lesion diameter in fruit at BR stage were more severe compared to fruit at MG stage. At 4 days of inoculation, disease incidence in control fruit at MG and BR stages reached 94.4 and 100%, respectively, with lesion diameter of 10.3 and 13.1 mm; in contrast, there were only 83.3 and 88.2% of disease incidence and 9.0 and 12.4 mm of lesion diameter in SA-treated fruits at the same maturity stages.

3.2. PR genes expression

The effects of SA or ET on transcriptional expression of *PR1*, *PR2* and *PR3* in tomato fruit at different maturities were shown in Fig. 2. The expression patterns of *PR1*, *PR2* and *PR3* in fruits at MG stage were apparently different from those at BR stage. SA significantly enhanced the expression level of *PR1* gene in tomato fruit at both MG and BR stages after 2 days of treatment, but had slight effects on the expression of *PR2* and *PR3* genes. By comparison, ET induced the expression of *PR2* and *PR3* in fruits at both MG and BR stages after treatment for 2 days, but had little effect on *PR1* gene.

3.3. Ethylene production

Generally, fruits at BR stage had higher ethylene content than at MG stage (Fig. 3). It was clear that SA treatment could effectively inhibit ethylene production in fruits at both MG and BR stages, resulting in lower levels of ethylene production than that of control until 3 days after treatment (Fig. 3). In contrast, ET treatment significantly stimulated ethylene production. After 1 day of treatment, a temporal burst of ethylene production was observed in ET-treated fruits at both MG and BR stages, in which levels were almost 4.6-fold in MG stage and 3.8-fold in BR stage, respectively, to control (Fig. 3).

3.4. SSC, pH and lycopene

There were no significant changes in lycopene content, SSC and pH in SA- and ET-treated fruits at MG stage after treatment. By contrast, SA-treated fruit showed lower lycopene content and pH value compared to ET-treated fruit at BR stage (Figs. 4 and 5). Lycopene content in fruit treated with SA at BR stage suppressed by more than 48% compared with the control; whereas fruit treated with ET at this maturity stage exhibited 25% higher of lycopene content than that in control (Fig. 4). Application of SA resulted in a decrease of pH value after 3 days of incubation, while ET increased the value immediately after treatment (Fig. 5D). SSC were not influenced either by ET or SA treatment in fruit at BR stage.

4. Discussion

Fruit maturity influences the natural resistance against fungal pathogens (Prusky, 1996). Therefore, to protect fruit from pathogen infection, it is necessary to investigate the mechanisms of fruit resistance at different maturity stages. Many hypotheses have been proposed to explain why ripened fruits are more susceptible to pathogens than young fruits. These include nutrient availability to trigger pathogen activation (Prusky, 1996), the ripening-related cell wall dissemble that facilitates pathogen expanding (Cantu et al., 2008) and the weakening of antioxidant defense response to accelerate cell death of host tissue (Chan et al., 2008). However, little attentions have been focused on the correlation of accumulation of pathogenesis-related (PR) proteins (PRs) with the acquisition of resistance in fruit at different maturity stages. PRs are defined as proteins which are induced in plant tissues in response to pathogenic attack or related stimuli. Based on the common biochemical and biological properties, PRs are divided into different families. Some of these, such as β-1,3-glucanases (PR2) and chitinase (PR3), have anti-fungal activity, but the biological function of *PR1* proteins remains elusive (van Loon et al., 2006b). Cota et al.
matic activities of varieties of tomato fruits, partly because of the changes in enzymatic activities of β-1,3-glucanase and chitinase in response to fungal infection. In the present experiment, we found that pretreatment with SA suppressed the decay of tomato fruits and induced the PR1 expression in fruits at both MG and BR stages; in contrast, ET reduced the disease incidence and activated the expression of PR2 and PR3 in fruits at both maturity stages. These results indicated that different PRs could be associated with different elicitor-induced fruit resistance. Our data were consistent with the notion that ET reduced the disease incidence and activated the expression of PRs in fruits at both maturity stages. These results indicated that different PRs could be associated with different elicitor-induced fruit resistance. Our data were consistent with the notion that PR1 was regulated by SA pathway, whereas PR2 and PR3 were corresponded to JA/ethylene pathway (van Loon et al., 2006b). Additionally, we found that application of exogenous SA and ET differentially regulates fruit ripening at BR stage, which might have an indirect effect on the fruit resistance to the invading fungal pathogens (Fig. 1). Our results suggest that there may be two mechanisms by which exogenous chemical compounds induce the fruit resistance against B. cinerea: the induction of PRs and the regulation of fruit ripening.

SA treatment was more effective in controlling decay caused by B. cinerea than ET in fruits at both MG and BR stages (Fig. 1). This effect may be attributed to the difference in the regulation of fruits ripening by SA and ET. Compared with controls, tomato fruit treated with SA showed the decrease of ethylene production and the decline of pH and lycopene content, whereas ET-treated fruit exhibited the increased levels of these factors, in BR fruits (Figs. 3–5). These results confirm the capabilities of SA and ET in suppressing and enhancing ripening process, respectively, in fruits (Zhang et al., 2003; Balbontín et al., 2007) and suggested that fruit qualities are more sensitive to hormonal treatment at BR stage than MG stage. It is well known that the susceptibility to pathogens is positively correlated with the degree of fruit ripening (Giovannoni, 2001); therefore, the inhibited ripening process observed in this study contributed to the suppression of decay in SA-treated fruits, whereas the accelerated fruit ripening in ET-treated fruits enhanced the sensitivity of tomato fruit to B. cinerea.

In conclusions, both SA and ET could induce resistance against B. cinerea infection in tomato fruits at both MG and BR stages. Their efficacies depended on the induction speed of PRs and the inhibition of fruit ripening. In fruits at MG stage, the resistance induced by SA or ET did not depend on the ripening factors, whereas in BR fruit, although more PRs were induced by SA and ET, the effects of SA and ET on ripening counteract the action of PRs in determining whether fruits were likely to be resistant or susceptible to pathogen.

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(2007) described that fruits at red ripe stage is more susceptible to Alternaria alternata than mature green stage in three different varieties of tomato fruits, partly because of the changes in enzymatic activities of β-1,3-glucanase and chitinase in response to fungal infection. In the present experiment, we found that pretreatment with SA suppressed the decay of tomato fruits and induced the PR1 expression in fruits at both MG and BR stages; in contrast, ET reduced the disease incidence and activated the expression of PR2 and PR3 in fruits at both maturity stages. These results indicated that different PRs could be associated with different elicitor-induced fruit resistance. Our data were consistent with the notion that PR1 was regulated by SA pathway, whereas PR2 and PR3 were corresponded to JA/ethylene pathway (van Loon et al., 2006b). Additionally, we found that application of exogenous SA and ET differentially regulates fruit ripening at BR stage, which might have an indirect effect on the fruit resistance to the invading fungal pathogens (Fig. 1). Our results suggest that there may be two mechanisms by which exogenous chemical compounds induce the fruit resistance against B. cinerea: the induction of PRs and the regulation of fruit ripening.

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