Application of Jasmonic Acid Followed by Salicylic Acid Inhibits *Cucumber mosaic virus* Replication

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Systemic acquired resistance is a form of inducible resistance that is triggered in systemic healthy tissues of local-infected plants. Several candidate signaling molecules emerged in the past two years, including the methylated derivatives of well-known defense hormones salicylic acid (SA) and jasmonic acid (JA). In our present study, the symptom on *Cucumber mosaic virus* (CMV) infected *Arabidopsis* leaves in 0.1 mM SA or 0.06 mM JA pre-treated plants was lighter (less reactive oxygen species accumulation and less oxidative damages) than that of the control group. JA followed by SA (JA → SA) had the highest inhibitory efficiency to CMV replication, higher than JA and SA simultaneous co-pretreatment (JA+SA), and higher than a JA or a SA single pretreatment. The crosstalk between the two hormones was further investigated at the transcriptional levels of pathogenesis-related genes. The time-course measurement showed JA might play a more important role in the interaction between JA and SA.

Keywords : Jasmonic acid, plant immune, salicylic acid, virus control

Salicylic acid (SA) and jasmonic acid (JA) are important signaling molecules in plant defense responses. The contribution of these signaling molecules in plant defense differs depending on the invading pathogen. For example, the SA-mediated defences have a major role in the basal resistance to the bacterial and Oomycete pathogens, *Pseudomonas syringae* and *Peronospora parasitica*, respectively, and also *Turnip crinkle virus* (TCV) and *Cucumber mosaic virus* (CMV) (Dong, 2001; Feys and Parker, 2000; Kachroo et al., 2000; Pieterse et al., 2001; Takahashi et al., 2002). In contrast, JA signaling has an important role in the basal resistance to the fungal pathogen, such as *Botrytis cinerea*

(Thomma et al., 1998). SA- and JA-dependent defense pathways have been shown to cross-communicate providing the plant with a regulatory potential to finetune the defense reaction depending on the type of attacker encountered. Induced resistance is regulated by a network of inter-connecting signal transduction pathways in which SA and JA function as key signaling molecules (Glazebrook, 2001; Pieterse et al., 1998; Reymond and Farmer, 1998; Thomma et al., 2001).

Our previous study showed that application of 0.06 mM JA and then of 0.1 mM SA 24 h later, enhanced *Arabidopsis*, tobacco, tomato and hot pepper resistance to CMV, tobacco resistance to *Tobacco mosaic virus*, and tobacco and tomato and hot pepper resistance to TCV. The inhibition efficiency to virus replication would usually achieve up to 80-90% (Shang et al., 2011). However, the putative molecular mechanism was not fully investigated. In this study, time-course expressions of SA/JA-related genes were detected. The measurement showed that JA may play a more important role than SA in this phytohormone interaction.

Materials and Methods

Plant culture and pathogen inoculation. All plants were grown in a temperature-controlled growth chamber with a 16 h-light 8 h-dark cycles at 20-25°C. The inoculation with virus was carried out as described previously (Shang et al., 2009). In brief, Carborundum was evenly applied to the surface of leaves, and virus, at a concentration of 1 µg/ml in 10 mM HEPES buffer pH 7.0, was rubbed onto the leaves with cheese cloth.

Before 24 h of virus inoculation, the seedlings were sprayed with JA (Sigma, St. Louis, MO, USA; methyl jasmonate could also be used and with the same concentration) followed by SA (Sigma). The optimal concentrations of JA and SA were 0.06 and 0.1 mM, respectively, and the optimal time for SA was 24 h after JA.

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RNA analysis. Total RNAs were isolated from plant tissues by Trizol reagent (Invitrogen). The RNAs were separated on 1.0% denaturing agarose gels. For RT-PCR, the first strand cDNA was prepared using the ReverTra Ace kit (TOYOBO). To further assay the expression levels of gene, quantitative real-time PCR analysis was performed on a Bio-Rad iCycler. *CMV-CP* was amplified with degenerate primers *CMV-F* (5'-CTGAACGGGTTGTCCATC-3') and *CMV-R* (5'-TGGTCTCCTTTWRGAGRC-3', R=AorG; W=AorT). *PR1* was amplified with primers *PR1-F* (5'-TCTTTGTAGCTCTTGTAGGTG-3') and *PR1-R* (5'-GATTCTCGTAATCTCAGCTCT-3'). *PR2* was amplified with primers *PR2-F* (5'-CTCTTTACAAACAACAAAAC-3') and *PR2-R* (5'-TTCATACTTAGACTGTCGAT-3'). *NPR1* was amplified with primers *NPR1-F* (5'-GATTCGGTTGTGACTGTTTTGG-3') and *NPR1-R* (5'-TCTCGTTTGTCTTCTTGCTCTA-3'). *WRKY53* was amplified with primers *WRKY53-F* (5'-CATCGCCAAGATTACATT-3') and *WRKY53-R* (5'-AGTCTGCGGCTACTACCA-3'). *WRKY70* was amplified with primers *WRKY70-F* (5'-AAAAGATTGGGACCCGTAA-3') and *WRKY70-R* (5'-TGGGAGTTTCTGCGTTGG-3'). *GST* was amplified with primers *GST-F* (5'-AGACTTTGGAGGGACGAG-3') and *GST-R* (5'-ACGGAGGACTACAAGAACTA-3'). *PDF1.2* was amplified with primers *PDF1.2-F* (5'-GCTAAGTTTGTCTCCATCAT-3') and *PDF1.2-R* (5'-CATGGGACGTAACAGATACAC-3'). *MPK4* was amplified with primers *MPK4-F* (5'-TATTCGCTCTAACCAACC-3v) and *MPK4-R* (5'-TCAACTGTGATGCGTCTG-3'). The cDNA was amplified by using SYBR Premix Ex Taq (TaKaRa). The amplification of the target genes was monitored every cycle by SYBR-green I fluorescence. The *Ct* (threshold cycle), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence was first detected, was used as a measure for the starting copy numbers of the target gene. Relative quantitation of the target gene expression level was performed using the comparative *Ct* method. Three technical replicates were performed for each experiment. *Actin1* gene was used as an internal control (F primer: 5'-CATCAGGAAGGACTTGACGG-3'; R primer: 5'-GATGGACCTGATCTGTCATAC-3').

Oxidative damage estimation. The H_2O_2 content of leaves was measured as described by Velikova et al. (2000). Approx. 0.5 g of fresh leaves were cut into small pieces and homogenized in an ice bath with 5 mL 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at $12,000 \times g$ for 20 min at 4°C. 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance of the supernatant was read at 390 nm.

Lipid peroxidation was estimated by measuring the thio-

barbituric acid-reactive substances (TBARS) as previously described (Xi et al., 2007). The lipid peroxides were expressed as TBARS content.

Electrolyte leakage was measured according to Cao et al. (2009). After measuring the conductivity, the Arabidopsis leaves samples were boiled for 15 min to achieve 100% electrolyte leakage.

Superoxide and H_2O_2 staining for *in situ* detection. *In situ* superoxide H_2O_2 were detected with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described previously (Shang et al., 2010). Arabidopsis leaves were excised at the base with a razor blade and supplied through the cut ends with NBT (1 mg/mL) or DAB (0.5 mg/mL) solutions for 8 h. Leaves were then decolorized in boiling ethanol (95%) for 15 min.

Results

The virus replication of Arabidopsis leaves infected with CMV. In the present study, Arabidopsis leaves were pretreated with 0.1 mM SA and 0.06 mM JA. Fig. 1 showed the *CMV-CP* level days after CMV inoculation. JA followed by SA (JA → SA) had the highest inhibitory efficiency to CMV replication (over 80%), higher than JA and SA simultaneous co-pretreatment (JA+SA), and than JA or SA single pretreatment. The expression levels of *CMV-CP* gene was further confirmed by the quantitative real-time PCR analysis (Fig. 1).

Superoxide and H_2O_2 staining. DAB staining confirmed the result that the lowest H_2O_2 accumulation occurred in

Fig. 1. *CMV-CP* gene expression levels of Arabidopsis systemic leaves infected with CMV. The *CMV* replication levels were detected by quantitative real-time PCR analysis at 0, 1, 3, 7, 15 days post inoculation. “JA → SA” indicates 0.06 mM JA followed by 0.1 mM SA pretreatment at the 24 h interval and before the virus inoculation at 24 h; “J+S” means JA and SA simultaneous co-pretreatment; “SA” indicates SA pretreatment alone; “JA” indicates JA pretreatment alone. Error bars show standard deviations ($n=3$).

Fig. 2. DAB and NBT staining of the CMV-infected Arabidopsis leaves.

JA → SA pretreated leaves, and lower than that of JA/SA single treated leaves or that of JA+SA treated leaves (Fig. 2). Corresponding to this result, accumulation of superoxide (stained by NBT) in JA → SA pretreated leaves was the lowest, when compared with other treatments (Fig. 2). The results suggested that JA → SA treatment was the most effective to CMV resistance.

Degrees of membrane injury. TBARS are one of the most important lipid peroxidation products. The TBARS content reflects the degree of membrane injury, and is an important injury indicator of plants under stress. The TBARS of the JA followed by SA pretreatment (JA → SA) was lighter than JA and SA simultaneous co-pretreatment (JA+SA), and than JA or SA single pretreatment (Fig. 3). H_2O_2 levels and electrolyte data reflected a similar trend (Fig. 3). The results suggest that virus infection reduces the stability and integrity of the plasma membrane, but the leaves pretreated with JA and SA were less affected.

Possible molecular pathways. The putative molecular pathways was investigated and some possible signaling factors have been defined. Transcripts of two transcription factors WRKY53 (Miao and Zentgraf, 2007) and WRKY70 (Li et al., 2004), MAP Kinase 4 (MPK4), JA-responsive resistance protein PDF1.2, SA-responsive resistance protein NPR1, PR1 and PR2 (Durrant and Dong, 2004; Spoel et al., 2003; Spoel et al., 2009) and ROS-related resistance protein glutathione S-transferase (GST) (Uquillas et al., 2004) were quantified in Arabidopsis seedlings inoculated with CMV (Fig. 4).

The expression level of *NPR1* inhibited by JA was de-

Fig. 3. Hydrogen peroxide, TBARS, and electrolyte leakage of the CMV-infected Arabidopsis leaves. Bars represent standard deviations of 3 independent replicates ($n=3$).

creased first and then increased. On the contrary, the expression level of *NPR1* was promoted by SA, and the level was gradually increased. For two hormones simultaneous co-treatment (JA+SA), in the case that SA works before JA, *NPR1* expression increased first and then decreased. For JA followed by SA treatment (JA → SA), JA works before SA, and the expression level of *NPR1* decreased and then increased to a relatively stable level. It was maintained at a suitable level at 15 dpi, but not increase dramatically during the infection. For CMV pure infection, the peak appeared at 7dpi and its expression increase dramatically.

The expression level of *PR1* was inhibited by JA, which decreased first and then increased. But the expression level of *PR1* was promoted by SA and was gradually increased then. The maximum peak appeared at 1 dpi. For JA+SA, in the case that SA works before JA, the expression level of *PR1* therefore increased first and then decreased. The peak appeared at 3 dpi. For JA → SA, JA works before SA, and the expression level of *PR1* was decreased first and then

Fig. 4. Defense gene expression levels in wild-type Arabidopsis seedlings were detected by quantitative real-time PCR analysis at -1, 0, 1, 3, 7, and 15 days post inoculation (dpi). “JA → SA” indicates 0.06 mM JA followed by 0.1 mM SA pretreatment at the 24-h interval and before the virus inoculation at 24 h; “J+S” means JA and SA simultaneous co-pretreatment; “SA” indicates solo SA pretreatment; “JA” indicates solo JA pretreatment. Error bars show standard deviations ($n=3$).

increased. The peak appeared at 1 dpi. It was lower than single SA treatment or two hormones simultaneous co-treatment, but higher than the pure virus infection (without any treatment) or sprayed with JA. The expression level of *PR1* induced by SA but repressed by JA was the maker of activation of the SA-mediated defense response against viruses. That means JA-mediated defense response plays a leading role in JA → SA. Similarly, the expression level of *PR2* fluctuated like *PR1*. Differently, the peak appeared at 7 dpi.

The expression level of *WRKY53* was inhibited by JA

which was decreased first and then increased. By contrast, the expression of *WRKY53* was promoted by SA which increased to the peak at 0 dpi and then decreased. For JA+SA, in the case that SA works before JA, the expression level of *WRKY53* was increased first and then decreased. For JA → SA, the expression level of *WRKY53* was decreased and then increased which made its level relatively stable, while the gene expression of other treatments had larger fluctuations, showing more than one peaks. For CMV pure infection, the peak appeared at 7 dpi and it shows only one peak.

The change of *WRKY70* expression level was similar to that of *WRKY53*. The only difference was that *WRKY70* expression level rose to its peak at 3 dpi.

ROS contribute to the symptom development and pathogenesis in compatible plant-virus interactions, as described recently in plum pox virus (PPV) susceptible peach plants (Hernandez et al., 2006). The expression level of *GST* in pure virus inoculation was increased continuously. The expression level of *GST* was enhanced by spraying JA alone which was higher than SA single treatment. For JA → SA, the *GST* expression level was the lowest. At 7dpi, the gene expression level reached to the peak and then increased to a relatively stable level.

The expression of *PDF1.2* was promoted by JA and the JA spraying alone made the level increased largest at 3 dpi. The expression was suppressed by spraying SA alone. For JA+SA, the peak appeared at 3 dpi, but was still lower than the JA → SA treatment.

The expression of *MPK4* was promoted by JA. The peak appeared by spraying JA alone at 1 dpi. For JA → SA, the peak appeared at 1 dpi. The expression of *MPK4* was inhibited by spraying SA. For JA+SA, the peak appeared at 3dpi, the same as for pure virus infection.

Among these, *WRKY53*, *WRKY70* and *PDF1.2* showed the maximum expression in the JA → SA pretreated seedlings at 10 dpi, compared with the other treatments, therefore relating to the optimal effect of JA → SA. *MPK4* transcript increased in all the seedlings inoculated with CMV. However, the increasing amplitude was the minimum in the JA → SA pretreated seedlings, compared with the other treatments.

Discussion

JA is a potential lipid-derived systemic acquired resistance (SAR) signal which might be an early signal establishing systemic immunity (Truman et al., 2007). Early accumulation of JA in phloem exudates and JA-dependent gene expression in the systemic leaves of infected plants correlates with SAR, while SAR is compromised in several JA signaling mutants. However, the link between JA and SAR remains unclear since SAR is not altered in all JA signaling mutants (Cui et al., 2005; Mishina and Zeier, 2007). Accumulation of SA is required for SAR, but only in the signal-perceiving systemic tissue: grafting experiments showed that tobacco leaves infected with TMV could transmit a SAR signal despite the presence of bacterial salicylate hydroxylase (SH) encoded by the *NahG* gene. By contrast, expression of this SA-degrading enzyme in systemic tissue abolished SAR signal perception (Vernooij et al., 1994). In many plant species, SA undoubtedly plays an important role in both locally expressed basal resistance and SAR

(Van Loon and Antoniw, 1982).

In tobacco, injection with SA elicited enhanced resistance to TMV, and application of SA triggered the accumulation of PR-proteins (Petersen et al., 2000), leading to speculation that SA might function as the endogenous signal. *NPR1* modulates cross-talk between SA and JA-dependent defense pathways. *NPR1* was active by SA treatment to suppress JA-dependent defense pathways (Spoel et al., 2003). SA suppresses jasmonate-dependent gene expression. The *WRKY70* transcription factor is a node of convergence for JA-mediated and SA-mediated signals in plant defense (Li et al., 2004). *MAPK4* negatively regulates SAR (Petersen et al., 2000). In wild-type plants, the defence genes were activated to regulate SAR. In other defective plants which could not produce SA or JA, the defense genes were also activated by treatment but the regulation of SAR was affected. These results suggested exogenous hormone must act through endogenous hormone.

Some genes were related to the antagonism of JA and SA. *PDF1.2*, *PR1*, and *PR2* were positive regulators of the antagonism of JA and SA. *WRKY70* and *NPR1* were negative regulator (Shang et al., 2011). These two genes maintain an endogenous JA and SA balance in their action. We found that if the genes expression induced by exogenous JA and SA was maintained in a suitable range, it takes advantage to the plant immunity. For JA → SA, the genes induced by JA gave faster responses, and the genes induced by SA were delayed. Therefore, it can be concluded that JA may play a more important role than SA in this phytohormone interaction.

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