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Full Length Research Paper

Role of calcium in tomato (*Lycopersicon esculentum* Mill.) resistance to *Botrytis cinerea*

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Tomato (*Lycopersicon esculentum* Mill.) was cultured in Hoagland's solution containing 0, 3 or 8 mM Ca^{2+}, to investigate the responses against *Botrytis cinerea* infection and the effects of Ca^{2+} on salicylic acid (SA) biosynthesis and signal transduction. The disease index was significantly lower in 8 mM Ca^{2+} treatment compared to the lower Ca^{2+} treatment groups. Gene expression studies exhibited that the expression level of chorismate mutase (CM), phenylalanine ammonia lyase (PAL) and activity of benzoic acid 2-hydroxylase (BA2H), which are involved in SA biosynthesis, were greater in the 8 mM Ca^{2+} group compared with the other treatments. This in turn, results in SA constitutive accumulation. Conversely, another SA biosynthesis pathway operates via isochorismate (ICS); the transcription of ICS-pathway related genes were hardly or only partly induced by the three treatment groups. The expression levels of SA-dependent signaling pathway genes (*NPR1, PR1, TGA2.2 and TGA1a*) increased significantly in the 8 mM Ca^{2+} treatment group after application of 2 mM SA, compared with the low Ca^{2+} treatments. Overall, all the changes were Ca^{2+} dose-dependent. The results suggest that Ca^{2+} had the ability to enhance SA accumulation and pathogen resistance with high concentration Ca^{2+} treatment; 8 mM Ca^{2+} also had a positive effect on SA-dependent signaling pathway in tomato seedlings.

Key words: Salicylic acid, Ca^{2+}, tomato, synthesis, signaling.

INTRODUCTION

Induced plant defense mechanisms involve phyto-hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene, and abscisic acid (Vlot et al., 2009; Van Loon et al., 2006; Asselbergh et al., 2008). SA acts as a signaling molecule in systemic immunity responses in plant defense, of which there are SA-dependent and SA-independent pathways (Malamy et al., 1990; Delaney et al., 1994; Cui et al., 2005).

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Abbreviations: SA, Salicylic acid; **PAL,** phenylalanine ammonia lyase; **ICS,** isochorismate; **CM,** chorismate mutase; **EDS1,** enhanced disease susceptibility 1; **PAD4,** phytoalexin deficient 4; **BA2H,** benzoic acid 2-hydroxylase; **NPR1,** non-expression of pathogenesis related gene 1; **SAR,** systemic acquired resistance; **PR1,** pathogenesis-related protein 1.

Generally, SA-dependent defenses are effective against biological pathogens, including biotrophic fungi and oomycetes, bacteria and viruses. The SA-dependent defense pathway is effective against *Oidium neolycopersici* in tobacco and against *Botrytis cinerea* in tomato (Achuo et al., 2004). Previous studies have shown that higher SA levels alone are sufficient to cause the expression of defense-related genes and to enhance the immune response (Santner et al., 2009). However, SA in plants can be generated via two pathways: i) SA synthesis via isochorismate in a two-step process involving isochorismate synthase (ICS) (also named SA induction deficient 2, SID2) and isochorismate pyruvate lyase (IPL) (Verberne et al., 2000; Wildermuth et al., 2001); ii) chorismate mutase (CM) can form SA in a pathway involving phenylalanine annomine lyase (PAL) and benzoic acid 2-hydroxylase (BA2H) (Wildermuth, 2006). Transgenic tobacco plants that overexpress

bacterial monofunctional ICS and IPL targeted to the plastid exhibit an SA-overproduction functional phenotype (Verberne et al., 2000). However, Wildermuth et al. (2001) showed that SID2 mutants, which are defective in ICS1, produce less SA than wild-type *Arabidopsis* plants. Suppressing the expression of *PAL* in transgenic tobacco plants leads to a decrease in SA accumulation in response to inoculation with tobacco mosaic virus (TMV) (Pallas et al., 1996). Exogenous application of 2-aminoindan-2-phosphonic acid, which is an inhibitor of PAL, blocks SA accumulation and suppresses pathogen resistance in *Arabidopsis* and potato (Coquoz et al., 1998; Mauch-Mani and Slusarenko, 1996).

The SA-dependent defense pathway has been well studied. SA-induced cytosolic non-expression of pathogenesis related gene 1 (NPR1) proteins change from oligomers to monomers, and subsequently, translocate into the nucleus from the cytosol (Dong, 2004; Mou et al., 2003; Despres et al., 2003). Then, NPR1 enhances the binding of transcription factors of the TGA family to regulate expression of defense genes such as *pathogenesis-related 1 (PR1)* (Zhang et al., 1999; Rochon et al., 2006). However, SA fails to activate PR1 expression or systemic acquired resistance (SAR) in *npr1* knockout mutants, suggesting that SA signaling requires functional NPR1 proteins (Zhou et al., 2000). Increased nuclear accumulation of NPR1 in the presence of SA is positively associated with increased *pathogenesis-related (PR)* gene expression and resistance to pathogens (Kinkema et al., 2000). Interaction between TGA2 and NPR1 is detected in the absence of SA, but it is enhanced by SA treatment (Lebel et al., 1998). Nevertheless, the ability of TGA2 to activate transcription requires both SA and NPR1 (Fan and Dong, 2002; Durrant et al., 2004; Rasmussen et al., 1991).

Calcium (Ca^{2+}) is a universal second messenger (Trewavas and Malho, 1998) and it has long been considered as the second messenger in many signaling cascades, including defense signaling (Wang, 2004; Sun, 2009; Lecourieux et al., 2006). In recent years, Ca^{2+} signa-ling has been intensively studied (Yang and Poovaiah, 2003), and a growing body of evidence points to the importance of Ca^{2+} in the synthesis of many hormones and in signal transduction. Recent studies have shown that binding of Ca^{2+}/CaM to the transcription factor SR1 in *Arabidopsis* represses the expression of *EDS1* and suppresses SA accumulation and resistance to *Pst* DC3000 (Du et al., 2009). Con-versely, binding of Ca^{2+}/CaM to microbe-associated molecular pattern (MAMP) -induced CaM-binding protein positively regulates SA accumulation and is involved in disease resistance against *Pseudomonas syringae* (Wang et al., 2009). In *Arabidopsis*, calcium-dependent protein kinases as a component of the innate immune system results in accumulation of SA and broad-spectrum pathogen resistance such as *B. cinerea*, *P. syringae* (Coca and Segundo, 2010).

These studies clearly demonstrate the link between Ca^{2+} and the accumulation of SA, SA signaling pathway and disease resistance in plants, but what is the mechanism by which SA synthesis occurs in response to calcium? Few studies have been undertaken to examine the effect of Ca^{2+} treatment on SA synthesis and signaling transduction.

The aim of this present study was to investigate whether Ca^{2+} affected SA biosynthesis and signal transduction, and thus, the activation of plant defense responses. Here, we provide evidence that calcium positively modulates the PAL-mediated pathway to increase SA content, which in turn induced pathogen resistance in tomato. Meanwhile, Ca^{2+} promoted the expression of SA-induced defense genes. These findings may be useful to exploit a broad-spectrum, efficient and nontransgenic approach to control plant resistance in the agriculture industry.

MATERIALS AND METHODS

Plant material, treatments and infection assays

Experiments were conducted in the solar greenhouse of the Science and Research Field of the Horticultural Department of Shenyang Agricultural University, P. R. China. The cultivated tomato hybrid (*Lycopersicon esculentum* cv. L402) was grown in plugs that contained a substrate composed of nutrient soil and vermiculite (2:1, v/v). Seeds were germinated at 28°C and 90% relative humidity. When the seedlings started to develop the fourth leaf, 30 seedlings of uniform size were transferred to each of four tanks (2 × 1.5 × 0.1 m). The tanks were filled with 70 L of half-strength Hoagland's solution and the tomato seedlings were allowed to adapt for five days. Then, seedlings were transferred to different treatment tanks: T0 (full-strength Hoagland's solution containing the usual 3 mM of Ca^{2+} $Ca(NO_3)_2$), T1 (full-strength Hoagland's solution adjusted with $CaCl_2$ solution to contain Ca^{2+} at 8 mM) and T2 (full-strength Hoagland's solution adjusted to contain 0 mM of Ca^{2+}: in case of Ca^{2+} absence in Hoagland's solution, 5 cm^3 of 1 M $NaNO_3$ /L Hoagland's solution was added in order to keep the balance in the nutrition solution). In one experiment, plants in the tanks were sprayed with 2 mM SA. Tissue samples were frozen using liquid nitrogen at different treatment times and stored at -86°C until used for SA biosynthesis and signal transduction analyses. The nutrient solutions were changed every five days and the levels adjusted every two days. Plants were grown in the greenhouse in natural light at 25 ± 1°C during the day and 15 ± 1°C during the night.

Inoculation and disease survey

Tomato seedlings were adapted in the tanks containing half-strength Hoagland's solution for five days, and these were then filled with different Ca^{2+} treatments of full-strength Hoagland's solution (as afore described) for three days. After this time, the seedlings were inoculated with *B. cinerea* spores by placing 5 ml of a suspension of 10^6 spores/ml^{-1} in 2% glucose solution. The disease survey was performed according to the methods of Fang (1998).

Determination of SA and salicylic acid SA-glucoside (SAG) contents

SA extraction was performed based on the methods described by

Yalpani et al. (1991). Briefly, each sample of 0.5 g was homogenized using liquid nitrogen and transferred to a 1.5 ml Eppendorf tube; 1 ml of 90% methanol was then added. The mixture was vortexed for 1 min, before sonicating for 2 min, and centrifuging for 5 min at maximum speed in an Eppendorf bench centrifuge. The supernatant was collected in a 2 ml Eppendorf tube, while the pellet was resuspended in 0.5 ml of 100% methanol. The sonication and centrifugation steps were repeated for this suspension. The supernatants were combined and re-centrifuged, before the solvent was evaporated in a SpeedVac concentrator at a high drying speed. Then, 250 µl of 5% trichloroacetic acid (TCA) was added to the residue, which was resuspended by vortexing. Partitioning was achieved by adding 800 µl of ethyl acetate: cyclohexane (1:1, v/v), which gave an upper phase of organic solvent containing free SA and a lower aqueous phase containing SAG. This partitioning was carried out twice. The combined upper layers containing the free SA were dried in a SpeedVac concentrator using a medium drying speed. The aqueous phase containing the SAG was subjected to acid hydrolysis by adding 300 µl of 8 M hydrochloric acid and heating the sample at 80°C for 1 h (Meuwly and Me´traux, 1993). After this, the acid fraction was partitioned again with the ethyl acetate: cyclohexane mixture and the procedure was continued as afore described. During the acid hydrolysis, SAG in the aqueous phase is converted into SA, and this was used to estimate the SAG content. SA was quantified by high-performance liquid chromatography (HPLC), as described previously (Marianne et al., 2002). Briefly, a Waters 600 E HPLC was used with a carbohydrate column and 2,410 refractive index monitors. The mobile phase was 75% acetonitrile in ultra pure water. The flow rate was 1.0 ml min^{-1} and the column temperature was35°C. Waters Millennium software was used to collect and analyze the data.

Extraction and analysis of BA2H

BA2H was assayed according to the method of Leon et al. (1993). Tomato leaf tissue samples of 0.5 g were frozen in liquid nitrogen and ground in a chilled mortar to give a fine powder. This was suspended in 1 ml of extraction buffer [20 mM Hepes (pH 7.0) containing 12.5 mM 2-mercaptoethanol, 10 mM sorbitol, 1% polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The suspension was vortexed, sonicated for 2 min, and then vortexed again before being centrifuged for 10 min at 10,000 ×*g*. The supernatant was used for the enzyme assays. All extraction procedures were performed at 4°C. The reaction mixture (final volume of 0.5 ml) contained 10 µM of Hepes buffer (pH 7.0), 1 µM of benzoic acid, 1 µM of NADPH and up to 200 µl of enzyme extract. The BA2H reaction mixture was incubated for 30 min at 30°C, and then 250 µl of 15% (w/v) TCA was added to stop the reaction. After vortexing and centrifuging for 5 min at 10,000 ×*g*, the supernatant was partitioned twice with 500 µl of ethyl acetate:cyclopentane: isopropanol (100:99:1). The upper organic phase was dried by vacuum centrifugation in an SVC 200 SpeedVac Sample Concentrator (Savant Instruments Co., Farmingdale, NY), and the pellet was resuspended in 150 µl of 55% (v/v) methanol. This methanolic solution was filtered through a 0.2-µm nylon filter, and SA was quantified by HPLC as afore described. BA2H activity was expressed as nM of SA formed h^{-1} g^{-1} fresh weight. The results did not change when activity was expressed as nM of SA formed h^{-1} mg^{-1} of protein.

RNA isolation and real-time polymerase chain reactions

Total RNA was isolated from treated leaves of tomato with the RNAprep Pure Plant Total RNA Extraction kit (Qiagen, Germany), followed by treatment with DNase I to remove any genomic DNA contamination. The quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed as described in Jain et al. (2006). In brief, cDNA samples were synthesized from 2 µg of total RNA using the High Capacity cDNA Archive kit (Applied Biosystems, USA). The cDNA was used as template and this was mixed with 200 nM of each primer and SYBR Green PCR Master Mix (Qiagen, Germany). qRT-PCR analysis was performed using the ABI Prism 7500 Sequence Detection System and Software (PE Applied Biosystems). Each primer pair (Table 1) was designed using Primer Express v.3.0 software (PE Applied Biosystems). The primer pairs were confirmed to amplify a unique and desired cDNA segment by basic local alignment search tool (BLAST) analysis of the tomato genomic sequence available in the NCBI database. The specificity of each reaction was verified by melting curve analysis. The relative mRNA levels for each of the genes in the RNA isolated from samples exposed to various treatments were quantified with respect to actin (the internal standard). At least, two independent RNA isolations were used for cDNA synthesis, with triplicate qRT-PCR analyses performed for each cDNA sample.

Statistical analysis

All data were analyzed using the OriginPro (OriginLab Corporation, Northampton, MA, USA) and the DPS v.3.01 statistical packages (Refine Information Tech, Hangzhou, Zhejiang, P. R. China). Measurements were performed on randomly selected samples from all three replicates of each treatment. Duncan's multiple range test at $P \leq 0.05$ or $P \leq 0.01$ was used to test for significant differences between treatment groups.

RESULTS

Enhanced resistance to *B. cinerea* in tomato treated with Ca^{2+}

Tomato was pretreated with different concentrations (0, 3 and 8 mM) of Ca^{2+} for threee days before inoculation with *B. cinerea* (Figure 1). The incidences of leaves and symptoms were examined at five days post inoculation, and the disease index was calculated. The disease index was significantly ($P<0.01$) lower in the 8 mM Ca^{2+} treatment group but significantly greater in the 0 mM Ca^{2+} treatment group compared with the 3 mM Ca^{2+} treatment group.

SA and SAG content

SA and SAG content increased from 0 to 6 h and the contents were the same at this time period in all treatments. In the 8 mM Ca^{2+} treatment group, SAG contents increased from 0 to 6 h after pretreatment, and remained relatively constant from 6 to 96 h (Figure 2). Free SA was also induced at 6 h, but to a lesser extent than SAG (Figure 2). The contents of free SA and SAG in the 8 mM Ca^{2+} group were about 2 to 2.5 fold higher than the other treatments at 24 to 96 h. However, SA and SAG levels in the 3 and 0 mM Ca^{2+} groups did not differ from each other

Gene expression of SA biosynthesis genes via two pathways in response to Ca^{2+}

There were no markedly significant differences in the

Table 1. Primers used for qRT-PCR.

Gene	Accession no.	Primer
EDS1	SGN-U564893	F 5'-AACAAGAGGGCTTTGCCTGAG-3' R 5'-GTGTCTTGGCAACAGGTGGA-3'
PAD4	SGN-U574519	F 5'-CCAAAAAGAGGGAATGGAGGTT-3' R 5'-TGTTCTCCCTTCATCATCATTTTTC-3'
PAL	M83314	F 5'-CAAGAATTATTTGATGCCTTAACCAA-3' R 5'-ACTATTCAAAAGGTCCATCAGTTTCC-3'
CM	SGN-U585231	F 5'-TTATCTATGGAGCTTGCACACCA-3' R 5'-GTTACGCATAATGAAAGGATGAAG-3'
ICS1	SGN-U572213	F 5'- GATTGACCAGTTAGTAGA -3' R 5'- GTTAGAGGAACATTTGAG -3'
NPR1	SGN-U585807	F 5'-GGTTTTGATGATAGCTTCTCCGATA-3' R 5'-ACGTCTTACGTTCAACTACTCCTTCA-3'
TGA2.2	SGN-U318626	F 5'-GTTTAGAAGGGTGAGTGATAC-3' R 5'-GTTTAGAAGGGTGAGTGACAT-3'
TGA1a	SGN-U570983	F 5'-AGCTCGTCATCACAACATACGC-3' R 5'-CAACTCGCCCTTGTCGATAAAC-3'
PR1	SGN-U318191	F 5'-GGCTATCCGTATGCCTCGTA-3' R 5'-CCACCCATCGGATGATTAAA-3
Actin	Q96483	F 5'-TGTCCCTATTTACGAGGGTTATGC-3' R 5'-AGTTAAATCACGACCAGCAAGAT-3'

qRT-PCR, quantitative real-time polymerase chain reaction.

expression of *EDS1* in the 0 to 8 mM Ca^{2+} groups during 0 to 72 h; but at 96 h, *EDS1* transcription levels increased rapidly in 0 mM Ca^{2+} treatment and was significantly higher than the other two treatments. Transcript levels of *PAD4* and *ICS1* were similar over time in the 8 and 0 mM Ca^{2+} groups; their levels reduced until they were lower than seen in the T0 group (Figure 3).

As shown in Figure 4, consistent with elevated SA levels, the expression of *CM, PAL* and BA2H activity (the BA2H gene has not been cloned in plants) are all at high levels in the 8 mM Ca^{2+} treatment after 6 h. The expression levels of *CM* were significantly higher in the 8 mM Ca^{2+} group compared with the other two treatments from 6 to 96 h; however, in 0 mM Ca^{2+} treatment, the level of *CM* was significantly lower than that level of 3 mM Ca^{2+} treatment after 24 h. PAL and BA2H increased obviously at/after 6 h in the 8 mM Ca^{2+} group and was markedly significantly higher than 0 and 3 mM Ca^{2+} treatment groups after 12 h. There were no markedly significant differences in BA2H activity between 0 and 3 mM Ca^{2+} treatment groups.

Effect of Ca^{2+} on expression of genes associated with the SA-dependent signaling pathway

As shown in Figure 5, on the basis of spray, 2 mM SA, 8 mM Ca^{2+} (T1+SA), 3 mM Ca^{2+} (T0+SA) and 0 mM Ca^{2+} treatments (T1+SA) had similar trends. *NPR1, TGA2.2, TGA1a* and *PR1* expression levels are induced and up-regulated, especially when 8 mM Ca^{2+} (T1+SA) was used; however, the 0 mM Ca^{2+} treatment (T2+SA) relatively slightly affected the transcript levels of the four genes, compared with the other two treatments. As shown in Figure 5a, the *NPR1* expression level in T1+SA treatment was greater than that seen in the other two treatments (T0+SA and T2+SA) from 6 to 96 h (Figure 5a); and the expression of *NPR1* in T0+SA treatment was significantly higher than T2+SA from 24 to 48 h. Similar

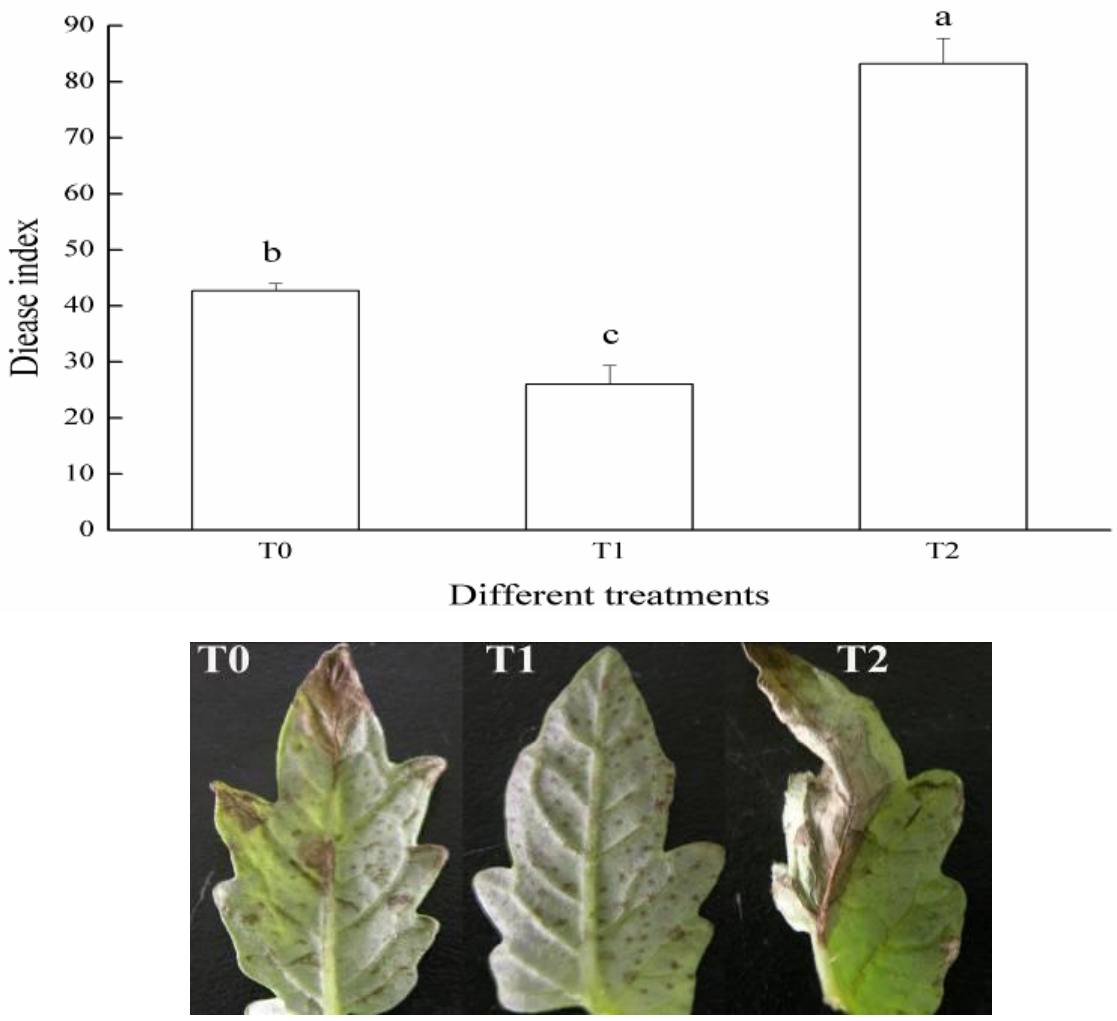

Figure 1. The disease index and symptoms of tomato leaves infected with *Botrytis cinerea*. Tomato leaves were pre-treated in different Ca^{2+} concentrations (T0, 3 mM Ca^{2+} as control; T1, 8 mM Ca^{2+}; T2, 0 mM Ca^{2+}) before *B. cinerea* inoculation. At five days post inoculation, the levels of incidence were surveyed and the disease index was calculated. Below each treatment, the typical symptoms are shown. Error bars show standard deviations (n = 3). Different letters indicate significant differences at $P \leq 0.05$ by Duncan's multiple range tests.

responses were observed with *PR1*, *TGA1a* and *TGA2.2* (Figures 5b, c and d), except at 48 h in *TGA2.2*, at 6, 48 and 96 h in *TGA1a*.

DISCUSSION

Our data shows that 8 mM Ca^{2+} treatment efficiency decreased the disease index compared with the 3 and 0 mM Ca^{2+} treatments after five days of inoculation with *B. cinerea* in tomato (Figure 1). These results support the conclusion that the application of calcium combination as growth media, reduces disease of plants attacked by foliar pathogens (Guillem et al., 2007); and applications of a few calcium compound solutions have reduced the severity of *B. cinerea* on paprika (Yoon et al., 2010). Consistent with the study of Wojcik and Lewandowski (2003), it was effective against *Botrytis* strawberry leaves led by $CaCl_2$ treatment, and Elad et al. (1993) obtained the result that *Botrytis* incidence was reduced to 50% in cucumber when Ca supplements were applied to the growth media.SA is an important hormone that plays a major role in plant defense responses (White, 1979; Malamy et al., 1990; Gaffney et al., 1993). Enhanced immune responses can be achieved by some chemical or pre-pathogen treatments - thanks to enhancement of endogenous SA levels (Durrant and Dong, 2004; Mauch-Mani and Slusarenko, 1996). Purified pyocyanin and chitosan can induce resistance to *B. cinerea* in bean and to powdery mildew in cucumber by enhancing SA levels (Kris-Etherton et al., 2002; Fan et al., 2010). SAR is induced in probenazole-treated *Arabidopsis* through the enhanced accumulation of SA (Yu et al., 2010). This study demonstrated that only 8 mM Ca^{2+} solution have

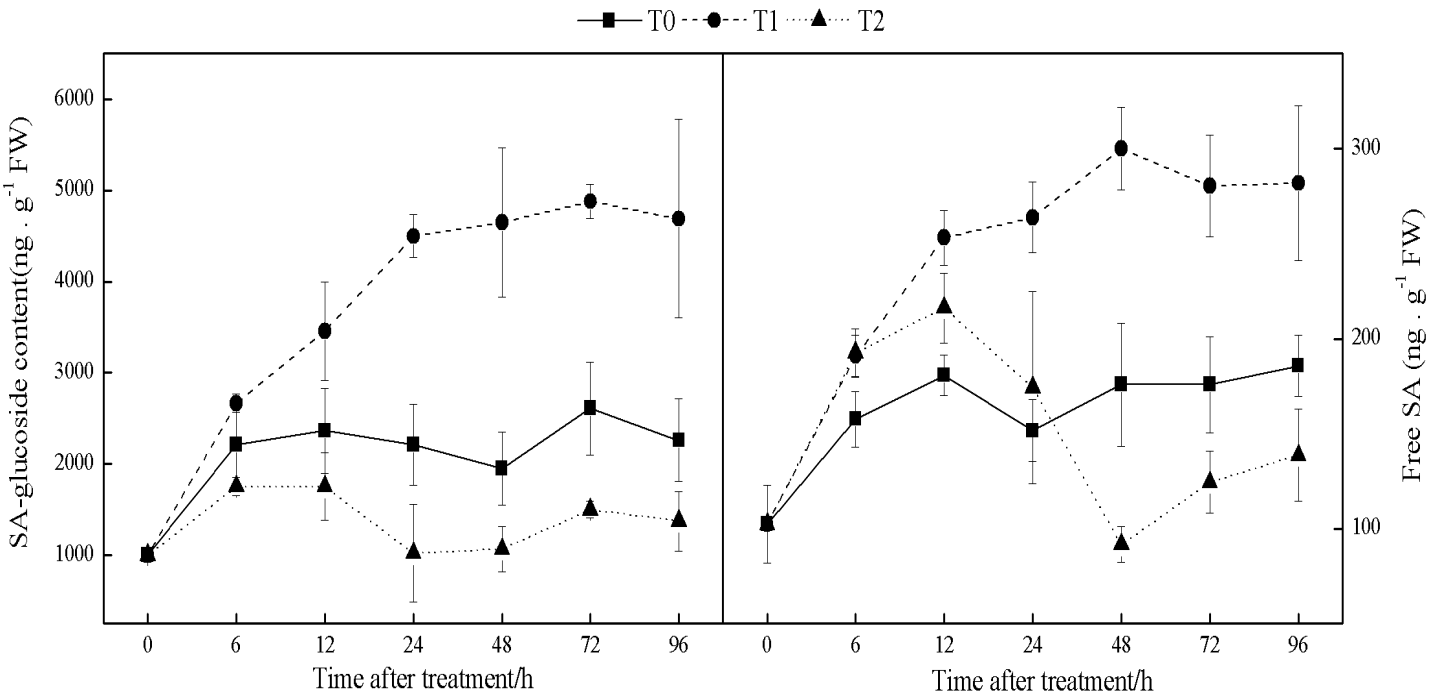

Figure 2. SA content of tomato leaves after different treatments concentrations of Ca^{2+}: T0, 3 mM Ca^{2+} as control (■); T1, 8 mM Ca^{2+} (●); T2, 0 mM Ca^{2+} (▲). A, Free SA content ; B, conjugated SA, SAG content. Error bars show standard deviations (n = 3). SA, Salicylic acid; SAG, salicylic acid B-glucoside.

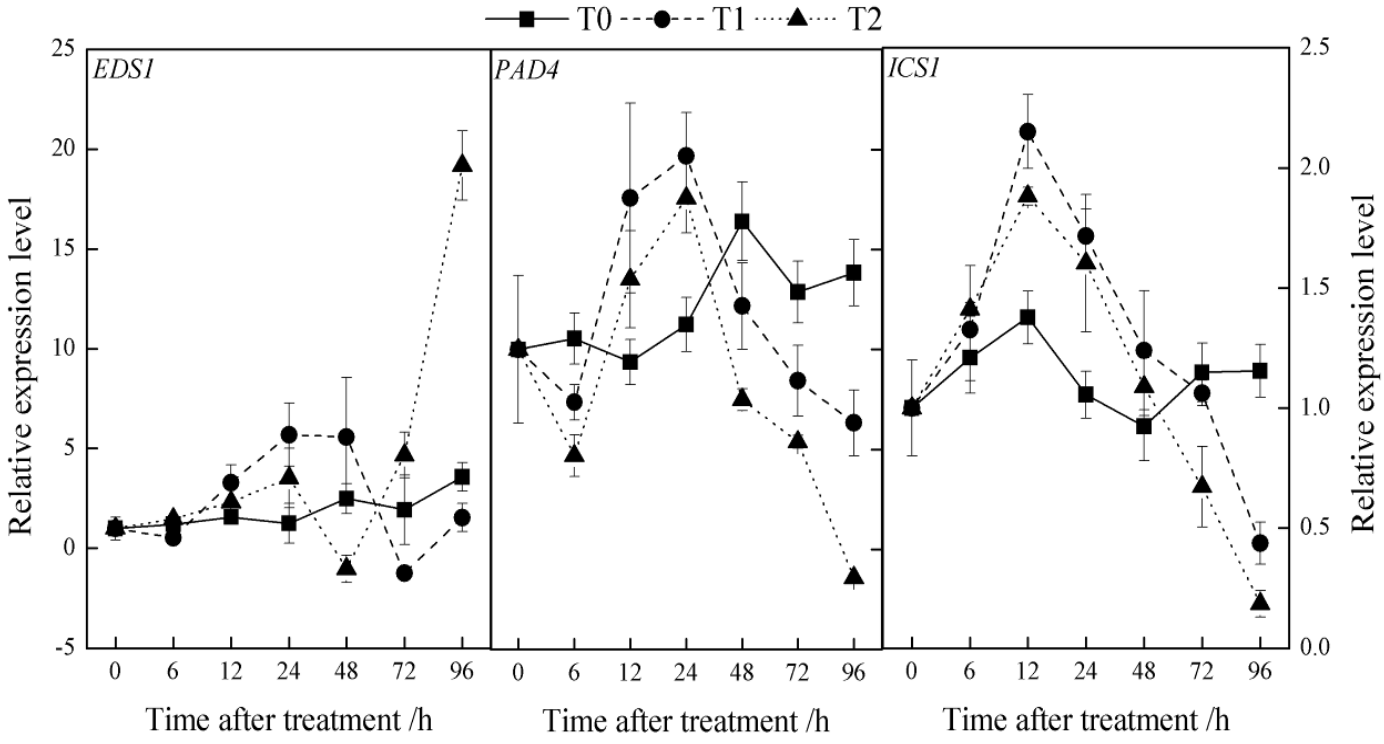

Figure 3. Expression of genes upstream of SA biosynthesis in the ICS-regulated pathway in different Ca^{2+} treatment groups. Gene expression levels were detected by qRT-PCR. T0, 3 mM Ca^{2+} as control (■); T1, 8 mM Ca^{2+} (●); T2, 0 mM Ca^{2+} (▲). The primers used are shown in Table 1. Error bars show standard deviations (n = 3). SA, Salicylic acid; ICS, isochorismate; qRT-PCR, quantitative real-time polymerase chain reaction

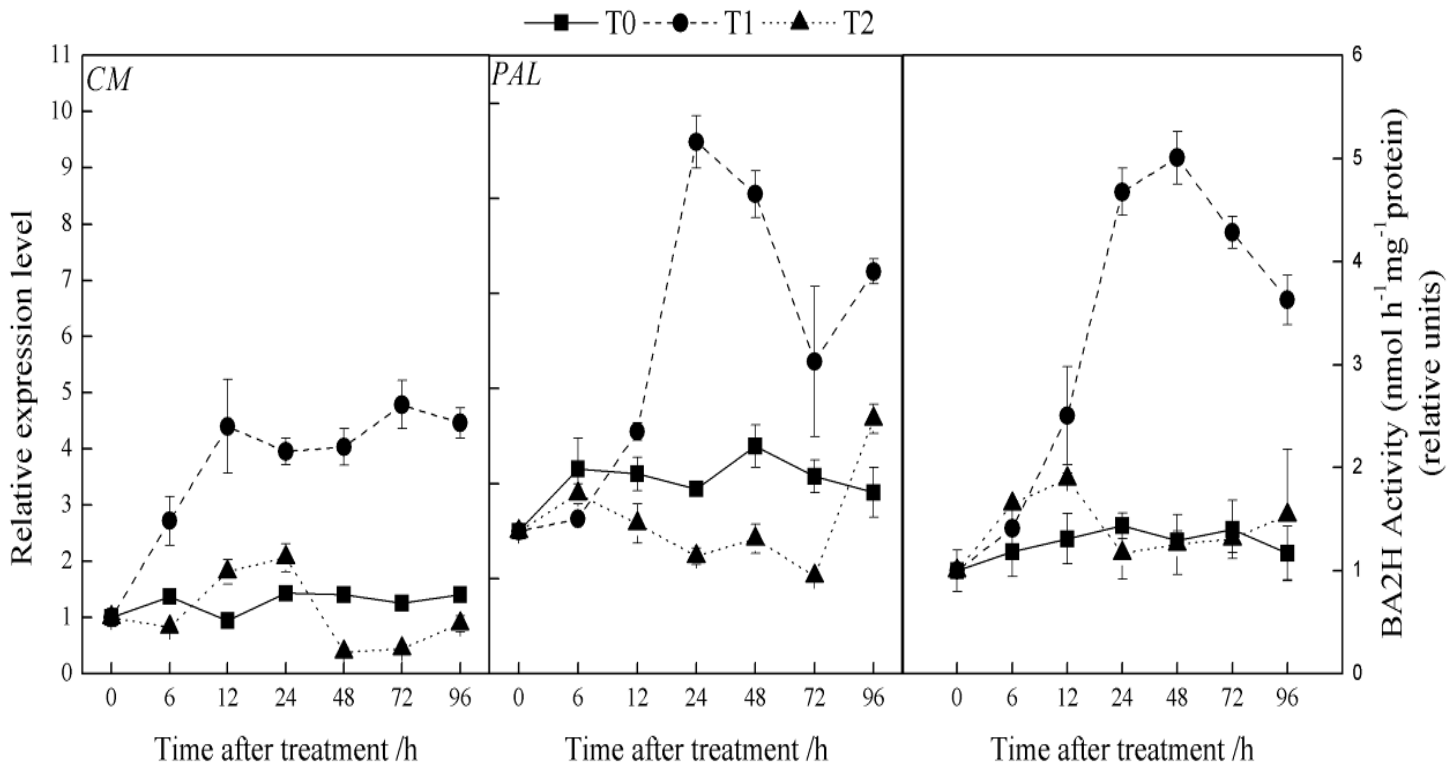

Figure 4. Gene expression and enzyme activity upstream of SA biosynthesis in the PAL- and BA2H- regulated pathway in different Ca^{2+} treatment groups. Gene expression levels were detected by qRT-PCR. T0, 3 mM Ca^{2+} as control (■); T1, 8 mM Ca^{2+} (●); T2, 0 mM Ca^{2+} (▲). The primers used are shown in Table 1. Error bars show standard deviations (n = 3). SA, Salicylic acid; PAL, phenylalanine ammonia lyase; BA2H, benzoic acid 2-hydroxylase; qRT-PCR, quantitative real-time polymerase chain reaction.

the ability to enhance the SA and SA-glucoside levels. Ca^{2+} is involved in many hormone syntheses (Trewavas and Malho, 1998; Sun, 2009; Wang, 2004; Yang and Poovaiah, 2003); it was reasonable to speculate that calcium could mediate SA-dependent defenses by a comprehensive and complex mechanism. Two major SA synthesis pathways in plants have been described: one is catalyzed by PAL, while the other involves ICS and IPL (Strawn et al., 2007; Verberne et al., 2000; Wildermuth et al., 2001). Both of the two SA synthesis pathways exist in tomato (Chadha and Brown 1974; Wildermuth et al., 2001; Uppalapati et al., 2007), but there is no consensus regarding which is the predominant pathway. However, the pathway for SA synthesis in response to Ca^{2+} has not yet been evaluated in tomato. In this present study, 0 and 8 mM Ca^{2+} treatments in ICS pathway *EDS1*, *PAD4* and *ICS1* gene expression levels were not markedly significantly difference before 72 h except *PAD4* at 6 h, and *ICS1* and *PAD4* sharply decreased; compared with 3 mM Ca^{2+} treatment at late time (≥72 h) (Figures 4 and 5), the differences were significant. The SA and SAG content did not decrease at the same time in 0 and 8 mM Ca^{2+} treatments. The *EDS1* expression level increased significantly after 72 h compared with 3 and 8 mM Ca^{2+} treatments. The 8 mM Ca^{2+} treatment significantly induced up-regulated transcript levels of genes and activity of enzyme under PAL pathway after 6 h. At the same time, these increases were concomitant with increasing levels of SA (Figure 2). PAL pathway related gene expression levels and enzyme activity treated with 8 mM Ca^{2+} were still higher than from those treated with 0 and 3 mM Ca^{2+}. The level is about two to three fold in 8 mM Ca^{2+} treatment compared to 3 mM; that was in accord with SA and SAG content in 8 mM Ca^{2+} treatment which was about two to three fold than that achieved with 3 and 0 mM. It indicates that the SA biosynthesis is mainly through PAL pathway and dose-dependency with different concentration Ca^{2+} treatment in tomato in our study.

SA-dependent defense pathway has been well studied (Dong, 2004). In *Arabidopsis*, NPR1 is required for positively regulating TGA2.2 to activate the expression of downstream defense genes, resulting in a final defense response (Thurow et al., 2005). *PR1* is a marker defense gene, which is an SA-induced marker gene. Downstream signaling in the SA pathway is largely regulated via NPR1 (Dong, 2004; Durrant and Dong, 2004; Pozo et al., 2004), which activates the TGA family and is SA-induced. NPR1 enhances the binding of basic leucine zipper protein transcription factors of the TGA family to the as-1 element

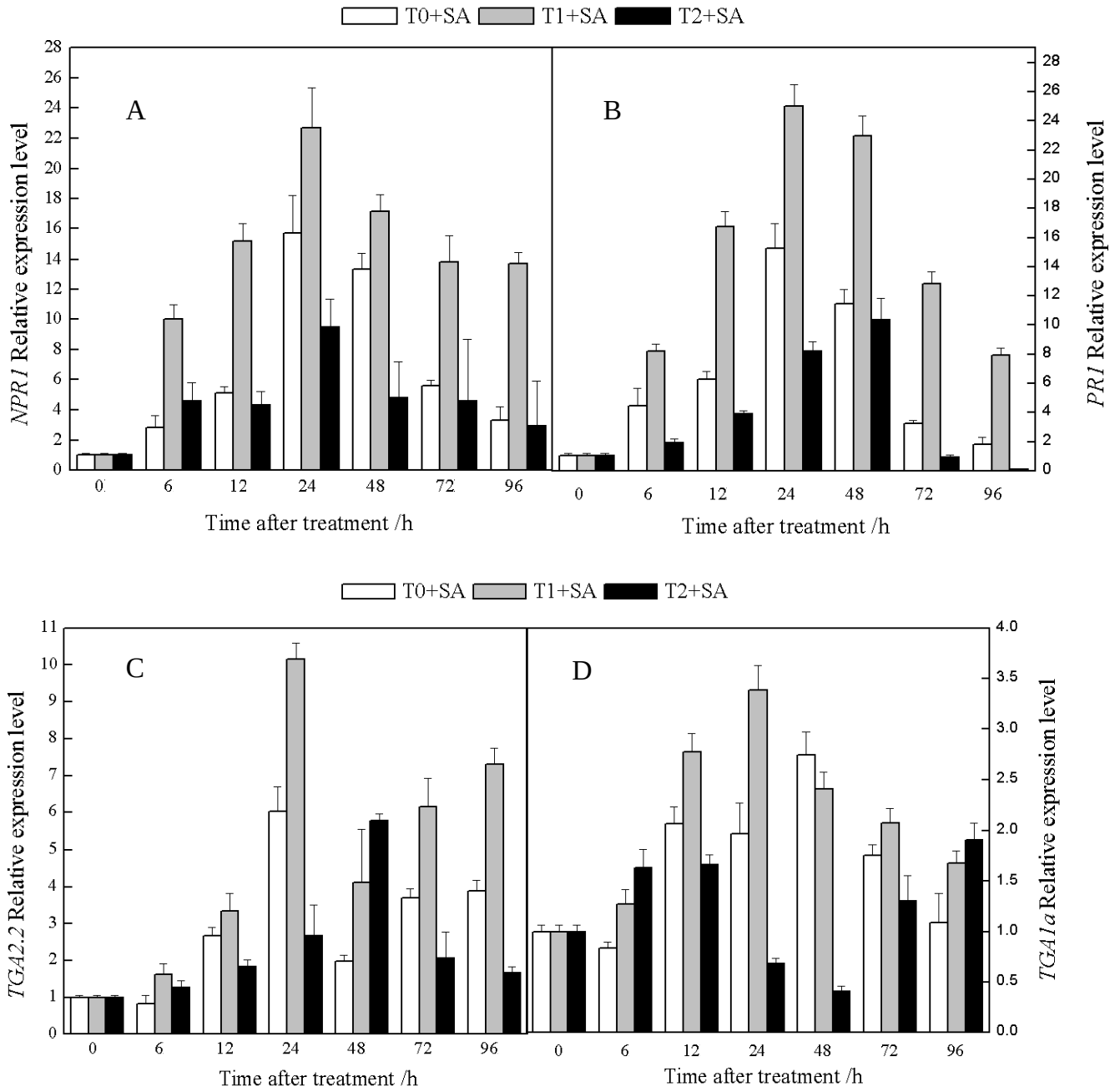

Figure 5. Effect of Ca^{2+} on the expression of SA-dependent signaling pathway related-genes. Gene expression levels were detected by qRT-PCR. The primers used are shown in Table 1. Error bars show standard deviations (n = 3). SA, Salicylic acid; qRT-PCR, quantitative real-time polymerase chain reaction.

of the *PR-1* promoter to activate *PR1* transcription (Lebel et al., 1998; Zhang et al., 1999; Zhou et al., 2000; Durrant et al., 2004). Consistent with our study, 2 mM SA enhanced the transcription levels of *NPR1, PR1, TGA2.2* and *TGA1a*, especially in 8 mM Ca^{2+} treatment which was in contrast to the treatment groups with no or low Ca^{2+} (T2+SA and T0+SA).

The results show a new finding that Ca^{2+} enhanced the SA-induced gene expression, and the trends showed a dose-dependency.

Conclusion

In summary, 8 mM Ca^{2+} is a safe, green and available precaution method for plant resistance, rather than precaution approach. Our results suggest that Ca^{2+} enhanced pathogen resistance in tomato seedlings. SA

biosynthesis may be mainly through the PAL pathway and dose-dependency with different concentration of Ca^{2+} treatment. Ca^{2+} was also involved in promoting SA-dependent signaling by inducing the expression of *NPR1, TGA2.2, TGA1a* and *PR1*. However, further studies should be conducted to clarify the different pathogens resistance treated with high Ca^{2+} cultivation and foliar application of Ca^{2+}. This could help producers to look for a broad-spectrum, efficient and nontransgenic approach to control plant resistance. This will also help the conductors to eliminate the suspicion of consumers caused by the suffering they pass through as a result of the food from transgenic plants.

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