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Inhibition effect on *tobacco mosaic virus* and regulation effect on calreticulin of oligochitosan in tobacco by induced Ca^{2+} influx

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ABSTRACT

Oligochitosan induce plant resistance against plant virus via different signal transduction pathways. Here, the Ca^{2+} signaling pathway was investigated by which oligochitosan elicited defense responses to *tobacco mosaic virus* (TMV) in tobacco (*Nicotiana tabacum cv.* Huangmiaoyu nn) plants. The results showed that the multiplication and movement of TMV in tobacco plants were inhibited by oligochitosan. Oligochitosan also increased cytosolic free calcium ions and regulated the expression of calreticulin. Furthermore, the inhibitory effect on TMV and the regulation effect on calreticulin were depressed when blocking the Ca^{2+} signaling pathway by EGTA. These results indicated that oligochitosan induced tobacco resistance to TMV through Ca^{2+} signaling pathway.

1. Introduction

Chitosan, as one of the most important elicitors, has been proved to elicit plant defense response to a broad spectrum of phytopathogens including plant virus (Chirkov, 2002; Terry & Joyce, 2004). In 1989, Pospieszny and Atabekov first reported that treatment of bean with chitosan decreased the number of local necroses caused by *alfalfa mosaic virus* (AMV) infection (Pospieszny & Atabekov, 1989). At present many experimental results have proved that chitosan can inhibit viral infection (Chirkov, Surguchova, & Atabekov, 1994; Pospieszny, 1997; Pospieszny, Chirkov, & Atabekov, 1991). These studies showed that chitosan treatment on plants could suppress virus infections regardless of virus types as well as plant species. However, the antiviral activity mainly depends on the molecular structure of chitosan, especially on the molecular weight. It was found that low-molecular-weight chitosan (oligochitosan) is much effective on suppressing infection of the *tobacco mosaic virus* (TMV) in tobacco plants, and antiviral activity of chitosan increased as its molecular weight decreased (Kulikov, Chirkov, Il'ina, Lopatin, & Varlamov, 2006). The efficient antiviral activity of low-molecular-weight fractions is presumably due to its better penetrating ability across the integuments of plants (Kulikov et al., 2006).

Although chitosan has been proved to be effective on plant resistance against plant virus, the mechanism still remains obscure. It was reported that chitosan-activated systemic acquired resistance against *tobacco necrosis virus* (TNV) ensued from a programmed cell death, which was similar to that occurred in the HR (hypersensitive response) (Iriti et al., 2006). Furthermore, chitosan application can also elicit callose deposition, which has a partial effect on inhibiting virus spreading (Iriti & Faoro, 2008). The activation of a Ca^{2+}-dependent callose synthase is one of the most rapid, effective cell responses to chitosan treatment (Köhle, Jeblic, Poten, Blaschek, & Kauss, 1985).

Calcium is one of the most important second messengers in numerous plant signaling pathways (Garcia-Brugger et al., 2006). In recent years, pathogen elicitors have been shown to induce changes in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$), although the temporal, spatial nature and the amplitude of $[Ca^{2+}]_{cyt}$ caused by different elicitors are diverse (Lecourieux, Mazars, Pauly, Ranjeva, & Pugin, 2002; White & Broadley, 2003). Many plant defense response are mediated by changes of $[Ca^{2+}]_{cyt}$. Subsequently plants protect themselves against pathogen infections (Grant et al., 2001; Hamel et al., 2005; Tada et al., 2001; Takezawa, 2000). In tobacco, laminarin sulfate induced the resistance against TMV infection after oxidative burst was Ca^{2+} dependent (Ménard et al., 2004). Lipopolysaccharides are able to promote plant disease tolerance through activation of Ca^{2+} signal (Gerber, Zeidler, Durner, & Dubery,

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2004). Chitosan, which has been shown to induce elevation of $[Ca^{2+}]_{cyt}$, activates plant defense responses through the calcium signaling pathway (Klüsenser et al., 2002). So Ca^{2+} is one of the early defense responses against pathogen infection.

Calreticulin (CRT), a multifunctional and evolutionarily conserved protein that exists in all eukaryotic cells investigated, plays a crucial role in Ca^{2+} storage and release, and regulates stress responses (Coppolino & Dedhar, 1998; Jia et al., 2008; Michalak, Corbett, Mesaeli, Nakamura, & Opas, 1999). CRT protein is located in endoplasmic reticulum (ER), one of the major Ca^{2+} storage organelles (Opas, Szewczenko-Pawlikowski, Jass, Mesaeli, & Michalak, 1996). It was reported that CRT was responsible for the main Ca^{2+}-retaining pool in ER. Increase of CRT led to increase of Ca^{2+} accumulation in ER-enriched fraction (Hassan, Wesson, & Trumble, 1995; Persson et al., 2001). CRT is also located in nuclear envelope, cell surface, plasmodesmata and so on (Gardai et al., 2005; Laporte et al., 2003; Napier et al., 1995). Moreover, CRT residing in plasmodesmata usually regulates plant virus movement (Boevink & Oparka, 2005). Chen also showed the colocalization and the interaction of CRT and TMV movement protein (MP), which consisted with Laporte's report on the interaction of CRT and *grapevine fanleaf virus* (GFLV) MP (Chen, Tian, Gafni, & Citovsky, 2005; Laporte et al., 2003). Furthermore, in transgenic plants, overexpression of CRT redirected TMV from plasmodesma to microtubules and compromised cell-to-cell transport of the virus (Chen et al., 2005).

Previously, the binding sites of oligochitosan (COS) in tobacco cells, the roles of signal molecules including NO and H_2O_2, and a protein kinase (*oipk*) have been investigated in COS-induced tobacco defense responses in our lab (Feng et al., 2006; Wang, Li, Zhao, Du, & Lin, 2008; Zhao, She, Yu, Liang, & Du, 2007a; Zhao, She, Du, & Liang, 2007b). In this study, the inhibitory effect of COS on TMV in *Nicotiana tabacum cv*. Huangmiaoyu nn was tested. Because of the importance of Ca^{2+} signal and CRT in plant resistance, we investigated the possible roles of Ca^{2+} and CRT in COS-induced tobacco plants resistance against TMV. These results indicated that oligochitosan induced tobacco resistance to TMV through Ca^{2+} signaling pathway.

2. Materials and methods

2.1. Plant materials

Tobacco (*N. tabacum cv*. Huangmiaoyu nn) was grown from seeds in a greenhouse and was used at the 4–6-leaf stage. Tobacco plants were kept in a growth chamber at $23 \pm 1\,^{\circ}C$ with a photoperiod of 16 h and 70–80% relative humidity for several days before treatments.

2.2. Preparation of COS

COS with 95% *N*-deacetylation and polymerization degree from 3 to 10 was self-prepared by enzymatic hydrolysis method, solubilized (50 mg/L) in deionized water (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999; Zhao, She, et al., 2007b).

2.3. COS treatment and TMV inoculation

Seedlings leaves of *N. tabacum* were sprayed, respectively, with 50 mg/L of COS, 0.05 mM abscisic acid (ABA), 2 mM EGTA, 50 mg/L of COS containing 2 mM EGTA and the control plants were sprayed with water (CK). 24 h after reagents application, plants were inoculated mechanically with TMV. Leave samples were collected at different time and frozen with liquid nitrogen for RNA isolation and protein extraction.

TMV used in our experiment was multiplied in *N. tabacum cv*. Huangmiaoyu nn. For mechanical inoculation, TMV was extracted from systemic infected tobacco leaves by homogenization in 0.05 M H_3PO_4 buffer (pH 7.0). After centrifugated at $2000 \times g$ for 6 min, the supernatant of extraction was used.

2.4. Observation of $[Ca^{2+}]_{cyt}$ by fluorescence microscope

$[Ca^{2+}]_{cyt}$ observation was performed by using Ca^{2+} fluorescent indicator fura-2/AM as described previously (Asai et al., 2008) with slight modifications. The epidermis was peeled carefully from leaves and cut into 5 mm length. Epidermal strips were incubated for 45 min in 20 mM Hepes buffer (pH 7.4, in dark) containing NaCl (115 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (0.8 mM), glucose (13.8 mM) and 1 μM fura-2/AM. The epidermal strips were subsequently washed three times with Tris/KCl buffer (Tris 10 mM and KCl 50 mM, pH 7.2). After washed the excess dye with fresh Tris/KCl buffer, the epidermal strips were placed in Tris/KCl buffer containing COS (50 mg/L, the same below), COS and EGTA (2 mM, Ca^{2+} chelator) or ABA (0.05 mM). Examination of peels was performed using fluorescence microscopy under the following settings: Excitation 330-400 nm, while monitoring emission at 510 nm. Images acquired from the fluorescence microscope were analyzed. The experiments were repeated at least three times in each treatment, and obtained the same results.

2.5. Total RNA extraction and RT-PCR analysis

Total RNA of tobacco was isolated using the TRIZOL reagent (Invitrogen, USA). The concentration and purity of RNA samples were determined by UV absorbance spectrophotometry.

For RT-PCR analysis, total RNA isolated from tobacco plants was reverse transcribed to first strand cDNA using oligo (dT) primer in a total volume of 10 μL according to the supplier's instructions (TaKaRa RNA PCR kit Ver.3.0). Resulting cDNA was then used as template for PCR amplification of *CRT* gene (GenBank Accession No. EU984501) in a volume of 25 μL as follows: 94 °C for 5 min; then 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s; and finally with an extension at 72 °C for 10 min, and then preserved at 4 °C. The tobacco *CRT*-specific primer pairs were designed according to CRTf 5′-TGGGAAAGCAGGTGGGTGA-3′, and CRTr 5′-AGGGCGGAGGATGAAAGTG-3′. The *actin* gene (GenBank Accession No. X69885) was used as positive internal control in a volume of 25 μL as follows: 94 °C, 5 min; then 30 cycles at 94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min; 72 °C, 10 min; preserved at 4 °C. The primer pair of actin was, 5′-GATGGTGTCAGCCACACTGTC-3′ and 5′-ATGCTGCTAGGAGCCAGTGC-3′, respectively. Amplified PCR products (10 μL) were electrophoresed on a 1.5% (w/v) agarose gel and monitored using the FR-980 Bio-Electrophoresis Image Analysis System.

2.6. Total protein extraction and Western-blot analysis

Leaf samples from healthy or TMV-infected *N. tabacum* plants were ground in liquid nitrogen and then dissolved in 500 μL extraction buffer (100 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 7.5% polyvinylpolypyrrolidone, 50 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM antipain, and 1 mM leupeptin). The extracts were then centrifuged at $13{,}000 \times g$ for 30 min and the protein concentration of supernatant was measured with BioRad kit. Equal amounts of supernatant protein were separated on 10% SDS-PAGE gels. Following electrophoresis, the presence of calreticulin or TMV CP (coat protein) was detected by Western-blot analysis using antibody against maize calreticulin (a generous gift from Dr. Jeff Gillikin, North Carolina State University, Raleigh, NC) or anti-TMV CP antibody (a generous gift from Dr. Zhou Tao, China Agricultural University), respectively.

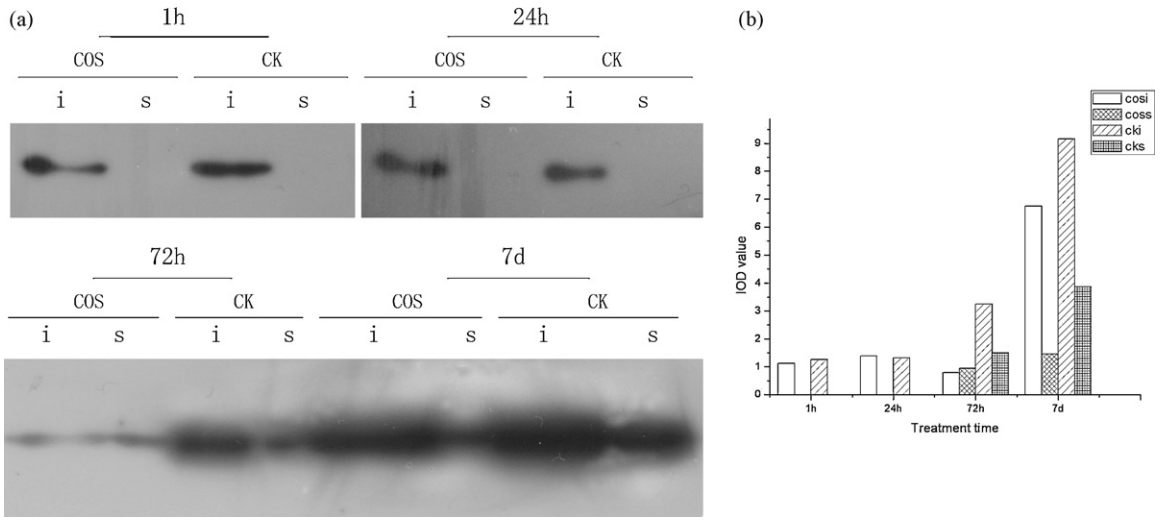

Fig. 1. Measurement of TMV CP content in inoculated leaves (i) and systemic leaves (s) of oligochitosan group (COS) and control group (CK) after TMV inoculation by Western-blot. TMV CP content was shown in this figure at different time after TMV inoculation. (a) The result of Western-blot. (b) The quantitative result of (a).

3. Results

3.1. Effect of COS on inhibiting TMV in N. tabacum

To study the effect of COS on inhibiting TMV in tobacco plants, we used TMV CP antibody to examine the TMV CP content in tobacco leaves. Total soluble protein was extracted from the inoculated leaves and the systemic leaves after TMV inoculation for 1, 24, 72 h and 7 days, respectively. As shown in Fig. 1, TMV CP was not detected in the systemic leaves regardless of the COS pretreatment or not in 24 h. 72 h after inoculation, the TMV CP content in inoculated leaves and systemic leaves of COS treatment group was less remarkably than that in control plants. The situation of the seventh day after inoculation was similar to that of 72 h, which indicated that more TMV assembled in CK group. The results indicated that COS pretreatment inhibited both TMV multiplication and movement.

3.2. Changes of $[Ca^{2+}]_{cyt}$ in epidermal cells of N. tabacum induced by COS

To confirm the role of $[Ca^{2+}]_{cyt}$ in COS elicited defense responses, the Ca^{2+}-sensitive fluorescent indicator Fura-2/AM was used. COS was found to enhance the level of intracellular Fura-2/AM fluorescence in tobacco epidermal cells, indicating COS-induced Ca^{2+} signal. Ca^{2+} mainly located at cell periphery (Fig. 2B), which was similar to ABA treatment (Fig. 2D). Meanwhile, the Fura-2/AM fluorescence indicating that Ca^{2+} signal was weak in the epidermal cells only loaded with Fura-2/AM (Fig. 2A). The results also showed that EGTA, the Ca^{2+} chelator could inhibit the level of Fura-2/AM fluorescence in the epidermal cells of *N. tabacum* leaves treated with COS (Fig. 2C), which suggested Ca^{2+} signal was inhibited by EGTA combined with Ca^{2+}.

3.3. Effect of COS on CRT expression

Considering the importance of CRT in TMV movement, CRT expression level of tobacco plants under different treatments (including COS treatment, TMV treatment, COS pretreatment before TMV treatment, and control) were investigated with by Western-blot method (Fig. 3A). The results showed that CRT expression was down-regulated after TMV inoculation or COS treatment, however, CRT expression increased in tobacco inoculated with TMV after COS pretreatment.

Since CRT protein expression level changed after COS treatment, the *CRT* gene regulation and CRT protein expression were investigated in detail. Total RNA and protein were extracted from *N. tabacum* seedlings treated with COS for 15 min, 1, 2, 4, 8, 12, 24, 48 and 72 h, respectively, and water treatment as control (CK). It was found that COS down-regulated *CRT* mRNA expression (Fig. 3B). Furthermore, COS inhibited expression of CRT (shown in Fig. 3C), and CRT expression was depressed gradually after COS treatment until 48 h.

After inoculation with TMV, CRT expression in tobacco leaves pretreated with COS was different from water pretreatment (CK) plants (Fig. 3A). To further analyse CRT expression difference between the two groups, TMV inoculated leaves and systemic leaves were investigated, respectively. As shown in Fig. 4, CRT expression in inoculated leaves pretreated with COS gradually heightened after TMV inoculation, which showed a time-dependent manner. However, the CRT expression in CK group was down-regulated, especially at early time of TMV inoculation, and the CRT expression was far less than that in the COS group. In the systemic leaves, CRT expression was up-regulated in both COS treatment group and CK group 4 h after TMV inoculation. And there were more CRT in systemic leaves than that in inoculated leaves. Through comparison of CRT expression in systemic leaves of two groups, it was found that CRT content in COS group was relatively higher than in CK group. All these results showed that COS pretreatment before TMV inoculation induced CRT expression in both inoculated and systemic leaves.

3.4. COS regulated defense response of tobacco to TMV and CRT expression by inducing Ca^{2+} influx

To understand COS-induced defense response to TMV, the tobacco plants were treated with water (CK), COS, COS and EGTA or EGTA as control. 72 h after TMV inoculation, TMV content was examined. TMV content reduced significantly in tobacco plants pretreated with COS for 24 h (Fig. 5A). But co-treatment with COS and EGTA blocked the reduction effect of TMV.

As CRT is a key protein interacted with Ca^{2+}, it was interesting to test the effect of co-treatment of COS and EGTA on CRT expression regulation. It was found that EGTA inhibited COS regulation on CRT

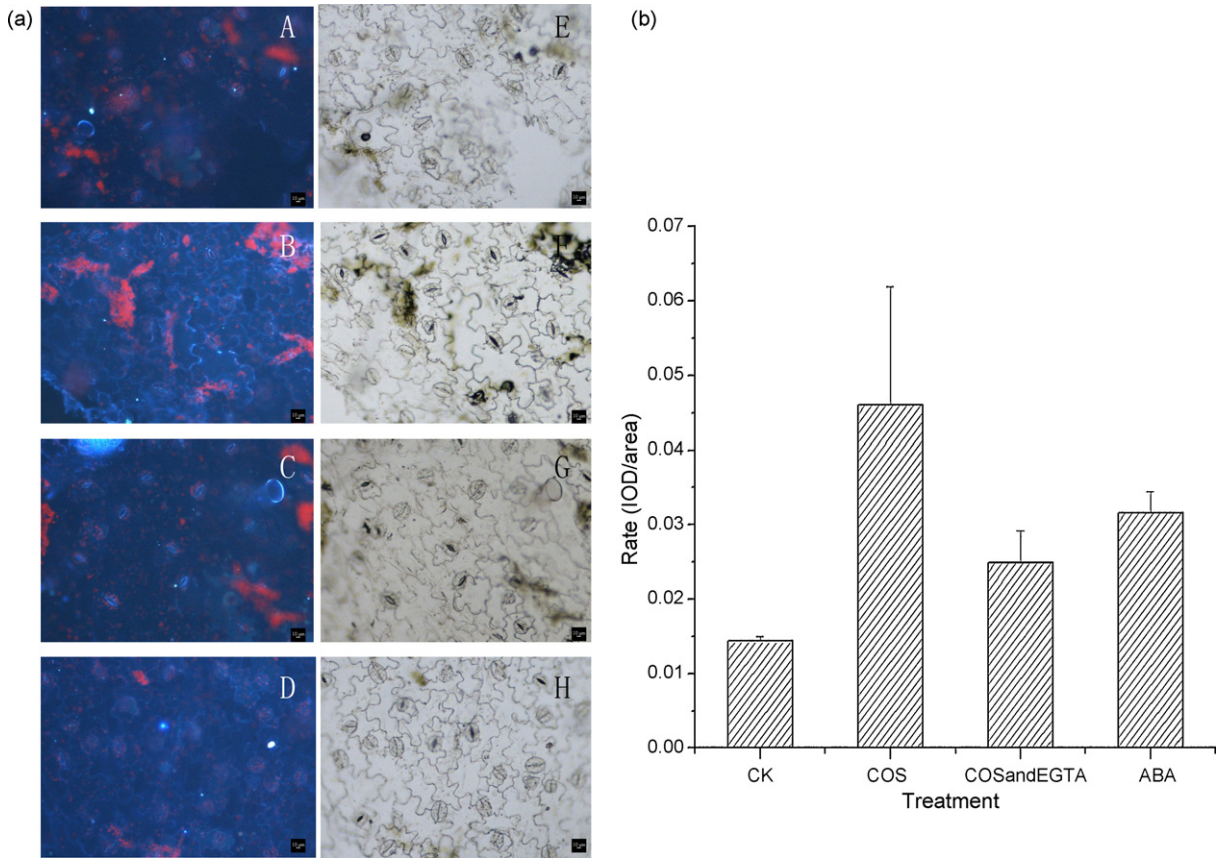

Fig. 2. Oligochitosan induced change of $[Ca^{2+}]_{cyt}$ in *N. tabacum*. (A) The cells loaded with fura-2/AM. (B) The cells loaded with fura-2/AM before treatment with oligochitosan. (C) The cells loaded with fura-2/AM before co-treatment with oligochitosan and EGTA. (D) The cells loaded with fura-2/AM before treatment with ABA. Images of (E)–(H) are bright images of (A)–(D), respectively. (a) The result of fluorescence photos. (b) The quantitative result of (a).

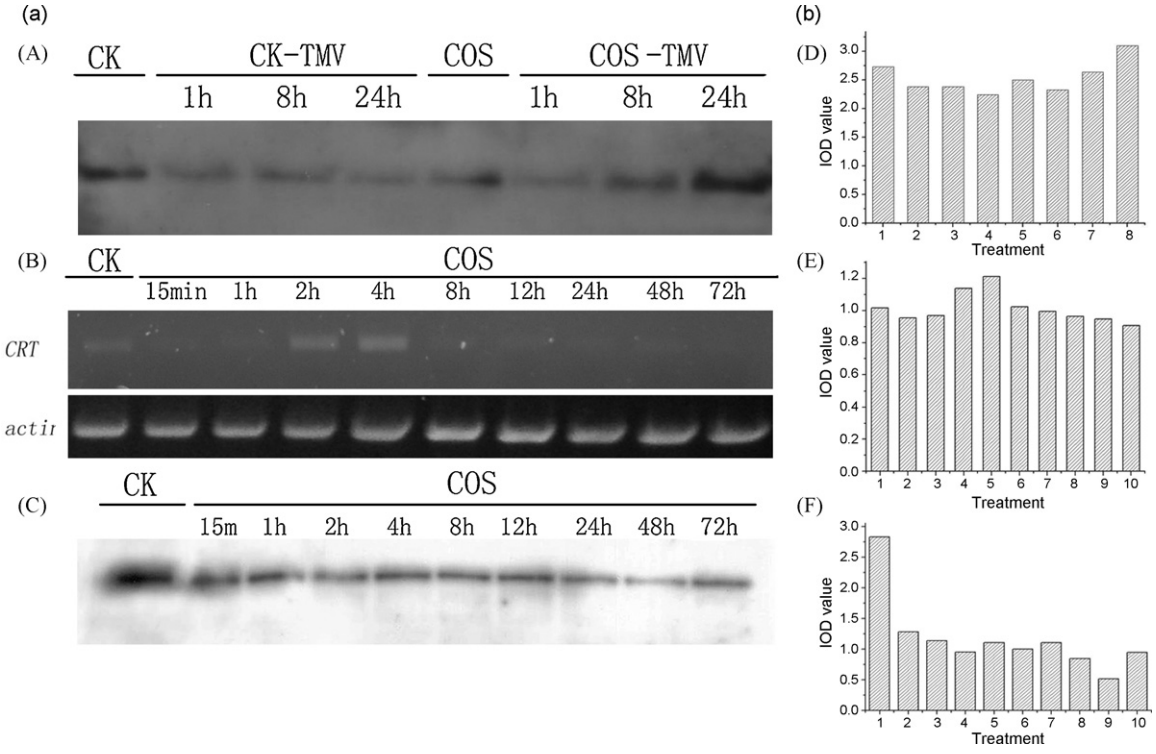

Fig. 3. Effect of different treatments on CRT in tobacco leaves. (A) Different treatment includes control, TMV treatment, COS treatment, and COS pretreatment before TMV treatment. Time showed in (A) means the time after TMV inoculation. CRT protein expression level was analyzed by Western-blot using anti-CRT antibody. (B) Effect of oligochitosan on *CRT* gene expression. mRNA was extracted and analyzed by RT-PCR and *actin* gene was used as inner control. (C) Effect of oligochitosan on CRT protein expression. CRT protein content was analyzed by Western-blot using anti-CRT antibody. (D) The quantitative result of (A). (E) The quantitative result of (B). (F) The quantitative result of (C).

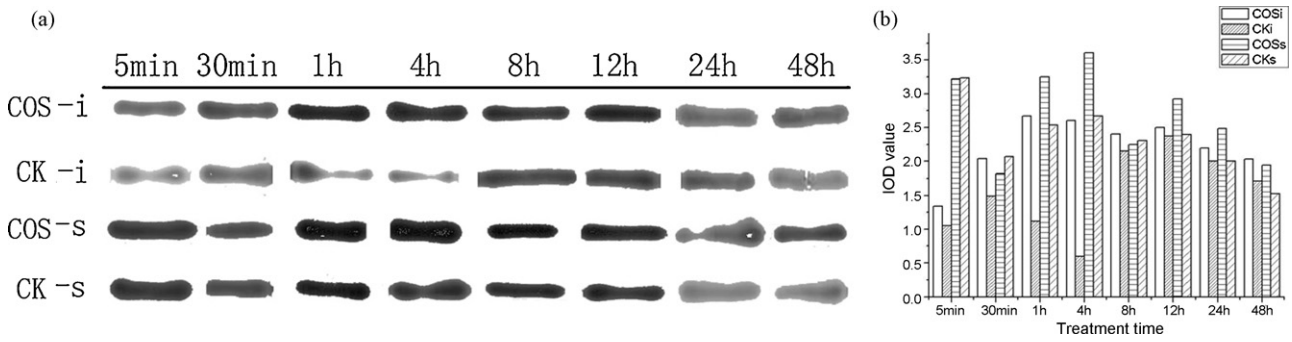

Fig. 4. Comparison of CRT expression in inoculated leaves (i) and systemic leaves (s) of oligochitosan group (COS) and control group (CK) after TMV inoculation by Western-blot. Time showed in this figure means the time after TMV inoculation. (a) The result of Western-blot. (b) The quantitative result of (a).

expression. In detail, 24 h after reagents treatment, EGTA blocked COS down-regulating CRT (Fig. 5B), while after TMV inoculation for 24 h, EGTA inhibited COS up-regulating CRT (Fig. 5C). At the same time, ABA treatment also decreased CRT expression, and only EGTA treatment was not different from CK group.

4. Discussion

In this study, COS-induced resistance against TMV was observed in *N. tabacum cv.* Huangmiaoyu nn which is susceptible to TMV (Fig. 1). Many experiments have showed that chitosan (including COS) induced plant resistance against viral disease in both resistant cultivars and susceptible cultivars, although the latter is not supported by certain *R* genes (Chen et al., 2009; Kulikov et al., 2006; Pospieszny & Atabekov, 1989). It is deduced that there are different mechanisms of defense responses induced by chitosan and COS in different plants. In the susceptible cultivars, it has been reported that the defense responses includes localized callose deposition in the plant cell wall and certain defense genes induction such as ribonuclease and β-1,3-glucanase (Chirkov et al., 2001; Iriti & Faoro, 2008). In this report, we found $[Ca^{2+}]_{cyt}$ and CRT induced by COS were correlative with plant defense responses.

Ca^{2+}, as an early signal molecular, participates in many defense responses induced by elicitors. Different calcium signatures depend

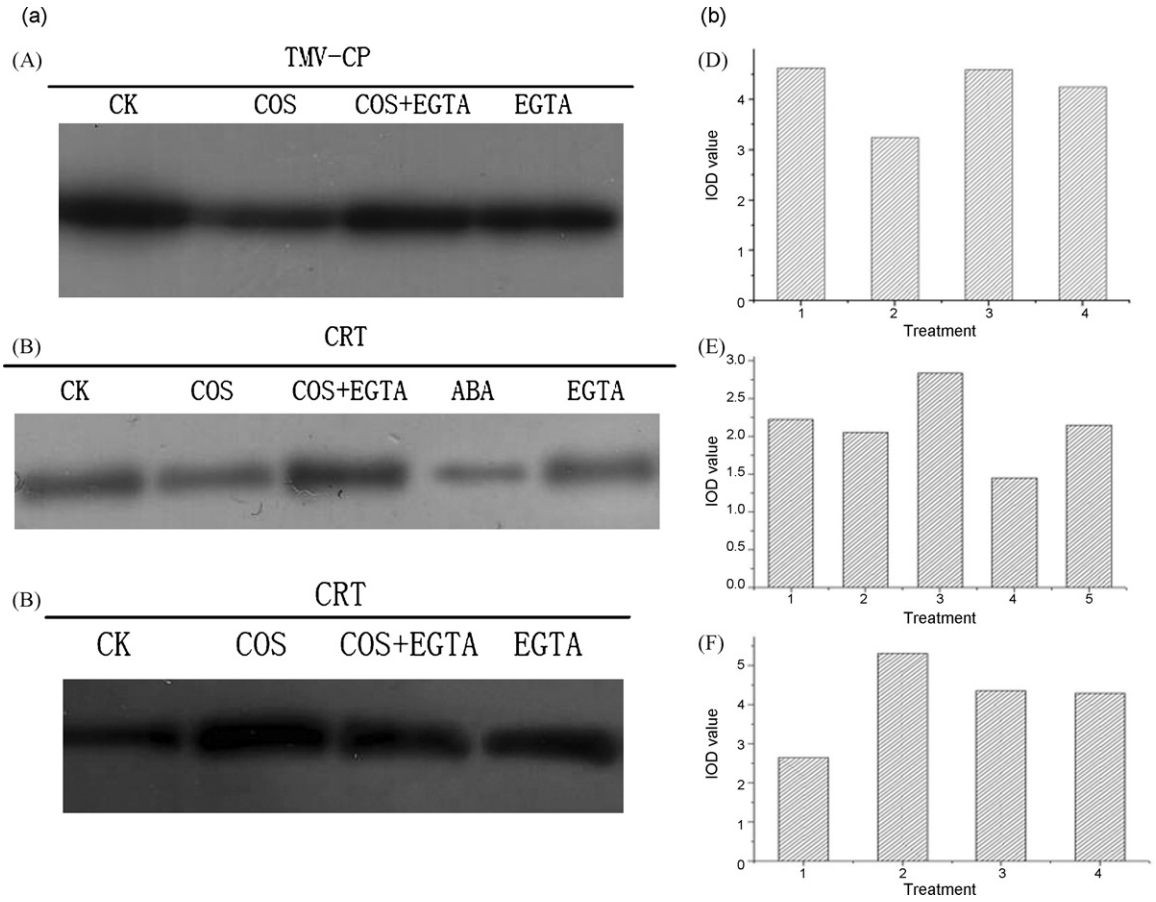

Fig. 5. Effect of TMV inhibitory and CRT regulation after blocking Ca^{2+} signal in oligochitosan inducing resistance process. (A) Measurement of TMV CP content in tobacco leaves of control group (CK), oligochitosan group (COS), and co-treatment of COS and EGTA, and EGTA after TMV inoculation 72 h by Western-blot. (B) Measurement of CRT expression in tobacco leaves of control group (CK), oligochitosan group (COS), and co-treatment of COS and EGTA, ABA and EGTA by Western-blot. (C) Measurement of CRT expression by Western-blot in tobacco leaves of control group (CK), oligochitosan group (COS), and co-treatment of COS and EGTA, and EGTA after TMV inoculation. (D) The quantitative result of (A). (E) The quantitative result of (B). (F) The quantitative result of (C).

on the elicitors or plants, including calcium transients, oscillations, or repeated spikes with specific subcellular location, lag time, amplitude and frequency (Gerber et al., 2004; Lecourieux et al., 2002). For example, chitosan was reported to elicit oscillations in Arabidopsis guard cells (Klüsenser et al., 2002). In soybean cells, chitin fragments caused a rapid increase in $[Ca^{2+}]_{cyt}$ (Mithöfer, Ebel, Bhagwat, Boller, & Neuhaus-Url, 1999). Our results showed that COS treatment elevated $[Ca^{2+}]_{cyt}$ in *N. tabacum* epidermal cells, and the induced $[Ca^{2+}]_{cyt}$ mainly located at cell periphery (Fig. 2B). The Ca^{2+} signal showed sustained increase in 1 h (data not shown), which was similar to ABA stimulation. Recently, it has been shown that extracellular callose deposition can constrain the cell-to-cell and long-distance transport of viral particles (Iriti & Faoro, 2008), and callose synthase is Ca^{2+}-dependent in chitosan treatment (Köhle et al., 1985). In this study, the resistance against TMV induced by COS decreased in the tobacco plants when extracellular Ca^{2+} was combined by EGTA (Fig. 5A). All results suggest that Ca^{2+} is concerned with plant defense response mediated by chitosan (including COS).

Increasing evidences indicate that CRT is involved in plant responses to a variety of stimuli. Our results showed that CRT was down-regulated by COS (Fig. 3C), which was consistent with ABA, brassinolide (BL), 6-benzyladenine (BA) regulation in rice (Komatsu, Jan, & Koga, 2009). Although the mechanism is not completely clear, it is speculated that COS arouses Ca^{2+} release from ER and the decrease of Ca^{2+} in ER results in the decrease of CRT expression. Similar to COS, exogenous ABA application also reduced TMV infection (Fraser & Whenham, 1982). Surprisingly, both elicitors could induce Ca^{2+} flux, CRT expression, callose deposition, stomatal closure and LEA protein expression, implied that the induction mechanism of the two elicitors may be analogical (Iriti & Faoro, 2008; Li et al., 2009).

CRT has been reported to localize to callose-enriched plasmodesmata and bind TMV MP to regulate TMV movement in tobacco (Baluška, Šamaj, Napier, & Volkmann, 1999; Chen et al., 2005). Overexpression of CRT results in mislocalization of TMV MP and impairment of TMV movement, although there are no reports on CRT depleted mutant and the exact function of CRT in TMV movement process. After investigation of CRT expression in TMV infection process, it was found that TMV inoculation decreased CRT expression (Fig. 3A). But there was more CRT expression in COS treated group than that in CK group (Fig. 4). This phenomenon probably resulted in partial restriction of virus movement. Meanwhile CRT expression regulated by COS was disrupted by EGTA, which indicated that COS regulation was mediated through the Ca^{2+} signaling pathway. As CRT combines with Ca^{2+} and maintains homeostasis of calcium level, it is necessary that Ca^{2+} and CRT are mutual regulated (Hassan et al., 1995).

Here, we reported the importance of Ca^{2+} in COS-induced defense responses to TMV infection in *N. tabacum*, and speculated on CRT regulation may contribute to resistance against TMV. But the mechanism of Ca^{2+} and CRT in plant defense responses is not clear. In the future, we will try to analyse the relationship between $[Ca^{2+}]_{cyt}$ concentrations and CRT expression, and construct *CRT* deletion mutant to clarify CRT location and function on plant defense responses. Then it will be helpful to understand the antiviral mechanism of COS on plant virus.

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