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Full Length Article

Function of Nitric Oxide in Chitosan Oligosaccharide-induced Resistance to Tobacco Mosaic Virus

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Abstract

Chitosan oligosaccharide (COS) has been shown to protect plants against viral invasion. To study its antiviral mechanism, nitric oxide (NO) signaling was investigated in the process of resistance to tobacco mosaic virus (TMV) in tobacco plants induced by COS. Results show that COS and NO donor sodium nitroprusside could induce NO generation quantified by Griess reagent in tobacco leaves with no TMV infection. However, the NO levels were not markedly different in various samples after TMV infection. The content of S-nitrosothiols, an important signal molecule downstream of NO was also determined, and results indicate that they may not be involved in the COS-induced antiviral process. In addition, western blotting experiments show that COS and sodium nitroprusside were observed to inhibit TMV multiplication and movement, which were blocked by NO scavenger cPTIO. COS and sodium nitroprusside-induced callose deposition were also reversed by treatment with cPTIO in aniline blue assay. The expression of β-1, 3-glucanase, which has been proved to control callose degradation, was regulated by COS and NO. Another protein, calreticulin related with TMV movement was also modulated by COS and NO. Collectively, these observations suggest that NO participates in COS-induced resistance to TMV by regulating calreticulin and β-1, 3-glucanase expression. © 2018 Friends Science Publishers

Keywords: β-1,3-glucanase; Callose deposition; Calreticulin; Chitosan oligosaccharide; Nitric oxide; Tobacco mosaic virus

Introduction

Chitosan oligosaccharide (COS) is an oligomer of chitosan that is obtained by deacetylation of chitin. As a plant resistance inducer, it has been widely used to elicit plant defense response and protect plants against pathogen invasion (Yin *et al.*, 2011; Ma *et al.*, 2013; Deng *et al.*, 2015; Li *et al.*, 2016). While the antivirus function has been reported in several plants induced by chitosan or COS (Rabea *et al.*, 2003; Zhao *et al.*, 2007; Iriti and Faoro, 2008), their antiviral mechanisms remain obscure. Most reports mainly focused on chitosan. The antiviral activity of chitosan depends on its degree of polymerization and N-deacetylation, the positive charge value, and molecular properties that modify the chitosan (Rabea *et al.*, 2003; Iriti and Faoro, 2008) as well as on their molecular weight and structure (Kulikov *et al.*, 2006; Davydova *et al.*, 2011). Chitosan could induce a similar response like hypersensitive response and stimulate callose deposition to inhibit virus spreading (Iriti *et al.*, 2006; Iriti and Faoro, 2008). Other reports found that chitosan oligomer could be bound to bacteriophage 1-97A particles, causing the direct DNA release and destruction of the phage particles (Kochkina and Chirkov, 2001). Hu *et al.* (2009) reported that the antiviral activity of guanidinylated chitosan was better than that of simple chitosan due to the direct alteration of the TMV configuration, congregation, and viral reduction. COS could also destroy TMV particles structure directly in a manner similar to that of chitosan (Shang *et al.*, 2008). However, there are few reports about COS-induced antiviral activity in plants. Zhao *et al.* (2007) found that the resistance of COS-induced tobacco to TMV was associated with NO pathway, although the function of NO in this process remained unclear.

Nitric oxide (NO) acts as a second messenger and plays a key role in plant and pathogen interaction (Delledonne *et al.*, 1998; Hong *et al.*, 2008). Many elicitors induce NO generation to regulate plant-pathogen response together with salicylic acid and jasmonic acid pathways (Klessig *et al.*, 2000; Zhou *et al.*, 2015). NO was also observed to modulate chitosan-induced resistance to blister blight in *Camellia sinensis* (L.) O. Kuntze (Chandra *et al.*, 2017). Our previous work reported the participation of NO in the process of resistance to TMV induced by COS in

tobacco (Zhao *et al.*, 2007). There are few reports on NO regulated plant-virus interaction. Fu *et al.* (2010) found that NO participated in defense response to viral infection in tomato by regulating AOX production and activity. Recently, more research is focused on the study of NO-related molecules, named as reactive nitrogen species, and the function of S-nitrosylation (Wendehenne and Hancock, 2011) in plant immunity. S-nitrosothiols is produced by the addition of NO to the -SH thiol groups present in proteins containing cysteine residues; it acts as an intracellular NO reservoir and plays a major role in plant immunity (Tada *et al.*, 2008; Yun *et al.*, 2016). It was found that S-nitrosoglutathione participated in plant immunity by facilitating NPR1 oligomerization through its S-nitrosylation (Tada *et al.*, 2008). However, there is no report about the role of S-nitrosothiols in COS-induced defense response.

Callose (β-1, 3-linked glucan) acts as a physical barrier to limit or prevent the spread of the virus from plasmodesmata or phloem. It has been found that callose deposition appears between the cell wall and plasma membrane to restrict pathogen infection (Li *et al.*, 2012; Ellinger and Voigt, 2014), and that callose metabolism is regulated by β-1, 3-glucanase. Studies of different plant species found β-1, 3-glucanase localized to extracellular spaces (Delp and Palva, 1999). There are three distinct classes of β-1, 3-glucanase reported in tobacco (Shinshi *et al.*, 1988) of which, class I was found to contribute to the defense against viral pathogenesis (Beffa and Meins, 1996). While the plant virus infection was partly inhibited in a β-1,3-glucanase-deficient mutant of tobacco (Iglesias and Meins, 2000), TMV mutants that over-expressed the β-1,3-glucanase gene spread faster through the cells in both wild-type plant and the β-1,3-glucanase deficient mutant (Bucher *et al.*, 2001). Calreticulin is an endoplasmic reticulum chaperone and is highly conserved in multi-cellular eukaryotes (Hassan *et al.*, 1995; Jia *et al.*, 2009) and plays a crucial part in Ca^{2+} homeostasis both in plants and animals (Del Bem, 2011). In plants, calreticulin is found in endoplasmic reticulum, nuclear envelope, cell surface, and plasmodesmata (Jia *et al.*, 2009). Studies prove that calreticulin has the same subcellular localization with TMV movement protein (Laporte *et al.*, 2003). Over-expression of calreticulin in plant leads to the redirection of TMV from plasmodesmata to microtubules, which inhibited the intercellular movement of the virus (Chen *et al.*, 2005). In our previous work we found NO and calreticulin to be involved in COS-induced antivirus response against TMV infection in *Nicotiana tabacum* var. Samsun NN and Huangmiaoyu NN (Zhao *et al.*, 2007; Lu *et al.*, 2010), nevertheless, the function and signaling pathway of NO in these processes are still obscure. The interactions among NO, β-1, 3-glucanase, and calreticulin are unknown. In this study, the role of NO in COS-induced resistance to TMV was investigated.

Materials and Methods

Chemicals

COS with 95% N-deacetylation and polymerization degrees from 2 to 9 was produced by enzymatic hydrolysis method and dissolved in distilled water (Zhao *et al.*, 2007). Sodium nitroprusside (SNP) and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3–oxide (cPTIO) were bought from Sigma. All other reagents were procured from Beijing Chemical Plant, Tianjin Kermel Chemical Development Centre, Beyotime Institute of Biotechnology, or Alfa Aesar.

Plant Materials

Tobacco (*Nicotiana tabacum* cv. Huangmiaoyu NN) seedlings were planted in a greenhouse at the temperatures of 26/16ºC with a photoperiod of 14/10 h and 63-81% relative humidity. In the condition of no TMV infection, healthy plants with four to six leaves were treated with 50 mgL^{-1} COS or water (control), and the leaves were collected at 0 h, 3 h, 8 h and 24 h for NO and S-nitrosothiols quantification. As for cPTIO treatment groups, leaves were pretreated with 100 μM cPTIO for 30 min and then sprayed with 50 mgL^{-1} COS, 100 μM SNP or water (CK). After all the treatments were done for 1 d, tobacco leaves were collected for NO, S-nitrosothiols and calreticulin identification. In case of TMV infection, tobacco leaves were pretreated with cPTIO (100 μM) for 30 min, then sprayed with 50 mgL^{-1} COS or 100 μM SNP for 1 d and were finally inoculated with TMV mechanically. Leaf samples, including inoculated TMV leaves and uninoculated TMV leaves were collected on 1 d, 7 d and frozen with liquid nitrogen for NO, S-nitrosothiols quantification, and protein extraction, and on 12 d for protein extraction and callose staining. TMV used in our experiment was maintained and extracted following the procedures and conditions as previously described by Lu *et al.* (2010).

Quantification of NO in Tobacco Leaves

The NO accumulation was measured by NO Assay Kit (Beyotime Institute of Biotechnology) as described previously by Zhang *et al.* (2011). Fresh leaf tissue (400 mg) was ground with 100 m*M* K-phosphate buffer (pH 7.0) and 0.6% (w/v) insoluble polyvinylpyrrolidone. The extract was treated with powdered activated carbon and centrifuged at 4°C for 10 min at 12400 rpm. The supernatant was filtered with a 0.22 μm PTFE Millipore membrane and assayed for NO immediately. Griess reagent I (50 μL) and Griess reagent II (50 μL) were added to 50 μL of the supernatant for 2 min at room temperature, and the absorbance was quantified by thermo multiskan ascent at 540 nm. A standard curve was prepared by different concentrations of $NaNO_2$.

Determination of S-nitrosothiols Content

S-nitrosothiols content was determined by the previously described procedure of Lindermayr *et al.* (2005). Fresh leaf tissue (200 mg) was homogenized with 100 m*M* Tris-HCl (pH 6.8) in a mortar. After centrifuging at 12800 rpm for 15 min at 4°C, the supernatant was filtered with a PTFE Millipore membrane (0.22 μm) and immediately assayed for S-nitrosothiols content. Briefly, 90 μL of sample was mixed with 15 μL of 0.5% ammonium sulfamate in water for 2 min. Then a total of 150 μL of 2.7% sulfanilamide and 0.25% $HgCl_2$ in 0.4 *M* HCl were added in the system, followed by 120 μL of 0.1% N-(1-naphthyl) ethylenediamine in water. The reference sample was under no $HgCl_2$. The concentration of formed azo compound was determined after 20 min by measuring the absorption at 540 nm. The S-nitrosothiols content was quantified according to a standard curve created with GSNO.

Callose Deposition

For callose deposition analyses, tobacco leaves with different treatments inoculated with and uninoculated with TMV at 12 d were stained with 0.05% aniline blue, and callose deposition was observed by epifluorescence microscopy as described by Ton and Mauch-Mani (2004).

Protein Extractions and Western Blotting

Tobacco leaves with different treatment were ground into powder in liquid nitrogen, and the powder was dissolved in 500 μL extraction buffer (100 m*M* HEPES, pH 7.5, 5 m*M* EDTA, 5 m*M* EGTA, 10% glycerol, 7.5% polyvinylpolypyrrolidone, 50 m*M* β-glycerophosphate, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* antipain, and 1 m*M* leupeptin). After the extracts were centrifuged at 13700 rpm for 30 min at 4ºC, the supernatants were transferred to fresh tubes and stored at –20ºC. Protein concentration was measured with the Bio-Rad protein assay kit. For western blotting, equal amounts of protein extracts (20 μg) were separated on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking in PBS-T (50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, and 0.05% Tween 20) with 5% nonfat dry milk for 2.5 h at room temperature, the membranes were incubated with polyclonal anti-TMV coat protein (TMV-CP) (from Dr. Zhou Tao, China Agricultural University), anti-calreticulin antibody (from Dr. Jeff Gillikin, North Carolina State University, Raleigh, NC) or anti-β-1,3-glucanase class I antibody (*Agrisera*) diluted with PBS-T at 4ºC overnight. After washing with PBS-T, the membranes were incubated with horseradish peroxidase–conjugated anti-rabbit IGg antibody diluted with PBS-T for 1 h at room temperature. The protein bands were analyzed by Image J.

Statistical Analyses

All the data in this paper were obtained from at least three independent experiments with three repeats, and the mean values ± SD are presented. Statistical differences in mean values at each point were determined with T test.

Results

COS Induces the Generation of NO and S-nitrosothiols

In healthy plants, the contents of NO in COS treatment groups were significantly higher than that in the control sample at 3 h, 8 h and especially at 24 h (Fig. 1a). COS and NO donor SNP induced NO production were decreased by treating with the NO scavenger cPTIO (Fig. 1b). In the TMV infection experimental groups (Fig. 1c), the NO content in inoculated TMV leaves (I) were higher than that in the uninoculated TMV leaves (U), however, NO content in both kinds of leaves showed no significant difference among different treatment groups (CK, COS± cPTIO and SNP ± cPTIO), while the NO content in the uninoculated TMV leaves was equal to that in leaves from healthy plants. Likewise, we determined the S-nitrosothiols content in above differently treated groups similar to NO (Fig. 1d, e and f). The content of S-nitrosothiols in COS treatment groups was slightly higher than that in the control groups (Fig. 1d), and in differently treated samples, S-nitrosothiols contents showed no significant difference from that in healthy plants (Fig. 1e). While after TMV infection, the trend of S-nitrosothiols content in different groups was observed to be similar with NO as shown in Fig. 1f. Our results indicate that S-nitrosothiols may be not involved in NO-mediated COS-induced resistance to TMV infection.

NO Participates in COS-induced Resistance to TMV Multiplication and Movement

To investigate the role of NO in COS-induced inhibition effect on TMV, tobacco leaves were first pretreated with 50 mg L^{-1} COS or other reagents for 1 d before inoculation with TMV. The TMV inoculated and uninoculated leaves at 1 d and 7 d were separately collected to identify TMV coat protein (TMV-CP) expression by western-blot. Results (Fig. 2) show that treatment with COS and NO donor SNP led to decrease in TMV multiplication in the inoculated TMV leaves at 1 d (1d-I), but no protein was detected in the uninoculated TMV leaves (1d-U), indicating the TMV has not moved to the uninoculated TMV leaves. But at 7 d, the TMV-CP was detected in both inoculated and uninoculated leaves, and the expression level of TMV-CP in COS and SNP groups were lower than that in other groups. cPTIO partly blocked the effects of COS and SNP, suggesting that NO participates in COS-induced resistance effect on TMV multiplication and movement.

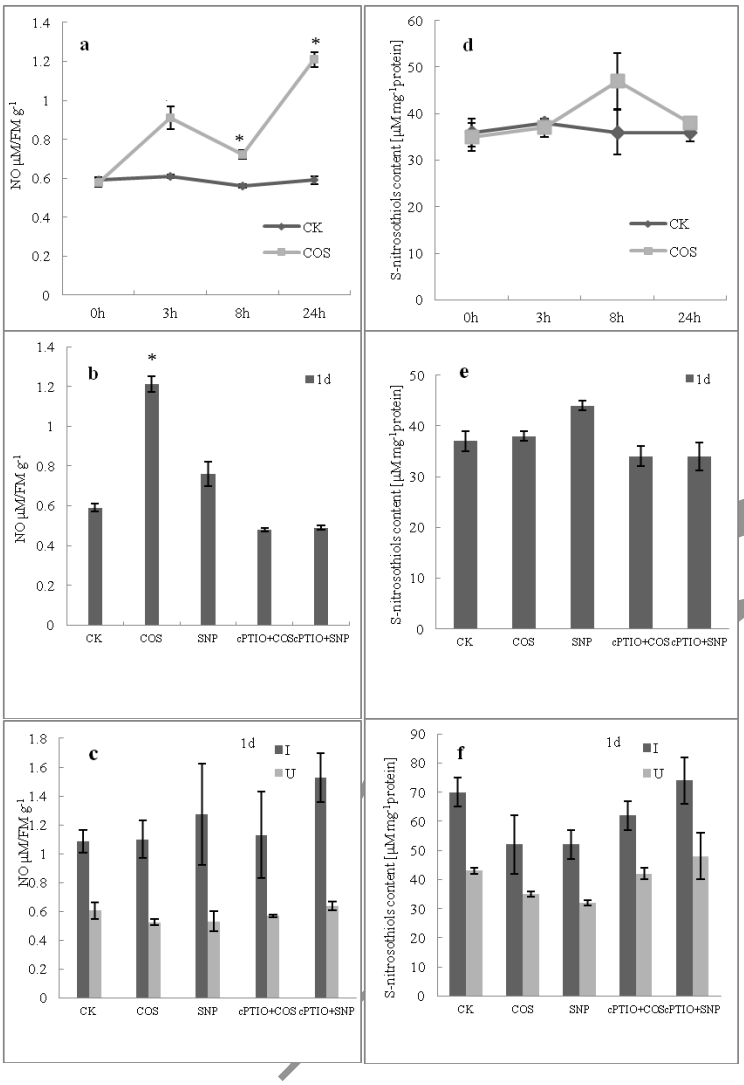

Fig. 1: Quantification of NO and S-nitrosothiols in tobacco leaves inoculated with or without TMV. (a) NO content in distilled water (CK) and COS (50 mg L^{-1}) treatment leaves at 0 h, 3 h, 8 h and 24 h without TMV infection. (b) Plants were sprayed with distilled water (CK), COS± cPTIO (100 μM) and SNP ± cPTIO (100 μM) for 1 d and the leaves were harvested for determining NO concentration without TMV infection. (c) NO content in tobacco leaves inoculated with TMV and uninoculated TMV leaves of different treatment groups at 1 d (see "Materials and methods"). I: inoculated TMV leaves; U: uninoculated TMV leaves. (d-f), S-nitrosothiols content in different groups with treatment similar as NO mentioned above. Data are presented as mean ± SD from three independent experiments. * $P < 0.05$ vs. control

NO Regulates COS-induced Callose Deposition and β-1, 3-glucanase Expression

Callose plays a critical role in preventing the spread of the virus, although there is no report whether COS can induce callose deposition in antiviral process. Thus, we first investigated callose deposition in tobacco leaves incubated with TMV by staining with aniline blue. As observed through fluorescence microscopy, COS and SNP could induce callose deposition in tobacco cell wall and stoma respectively (Fig. 3a) only in the inoculated TMV leaves (I), and cPTIO could reverse this effect (Fig. 3b). In the uninoculated TMV leaves (U), no callose deposition was detected in all the experimental groups. The results suggest that COS regulated callose deposition is mediated by NO signal.

To investigate how NO regulates COS-induced callose deposition, we studied the expression of β-1, 3-glucanase in tobacco leaves. As shown in Fig. 3c, the β-1, 3-glucanase expression was up-regulated by COS and SNP in the inoculated TMV leaves at 12 d and down-regulated in the uninoculated leaves, while cPTIO treatment led to inhibiting effect on COS and SNP, suggesting NO could regulate β-1, 3-glucanase expression. However, our results about β-1, 3-glucanase expression were contradicted with callose deposition in tobacco leaves,

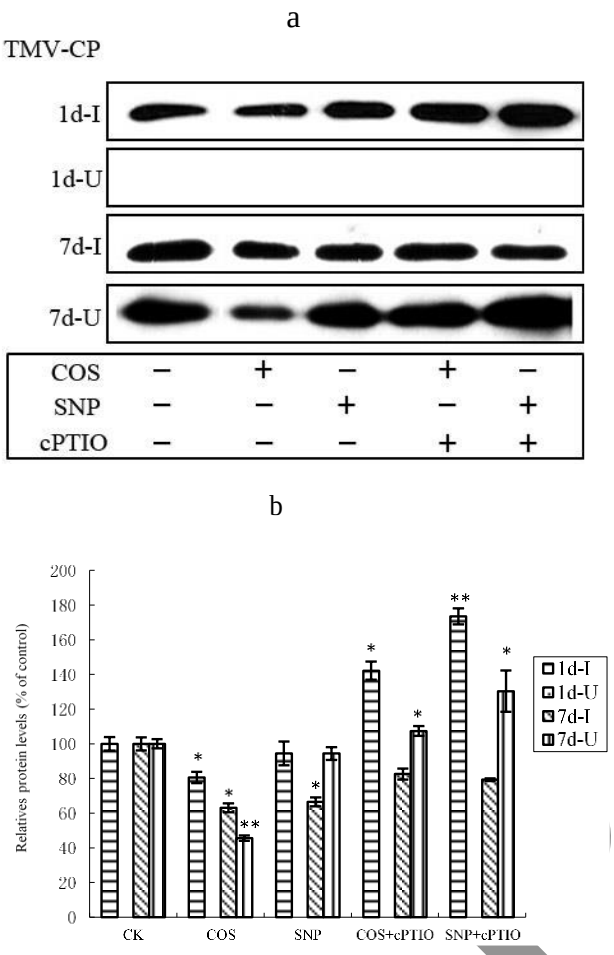

Fig. 2: TMV-CP content was determined in inoculated TMV leaves and uninoculated TMV leaves of different groups after TMV inoculation at 1 d and 7 d by western-blot. (a) The result of western-blot. (b) Quantitative determination of result obtained in (a). I: inoculated TMV leaves; U: uninoculated TMV leaves. Data are presented as mean ± SD from three independent experiments. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control

and the reasons need to be further studied. Thus, the results suggested that NO regulates COS-induced callose deposition, which may be related to β-1, 3-glucanase expression.

NO regulates COS-induced Calreticulin Expression

To test how NO took part in COS-induced resistance to TMV cell-to-cell movement, the calreticulin expression was determined in tobacco leaves. Calreticulin expression increased in the presence of COS and SNP and decreased by treating with cPTIO at 1 d in the TMV inoculated leaves and at 7 d in both kinds of leaves, although, no significant difference in calreticulin expression was observed in these groups at 1 d in the uninoculated leaves (Fig. 4).

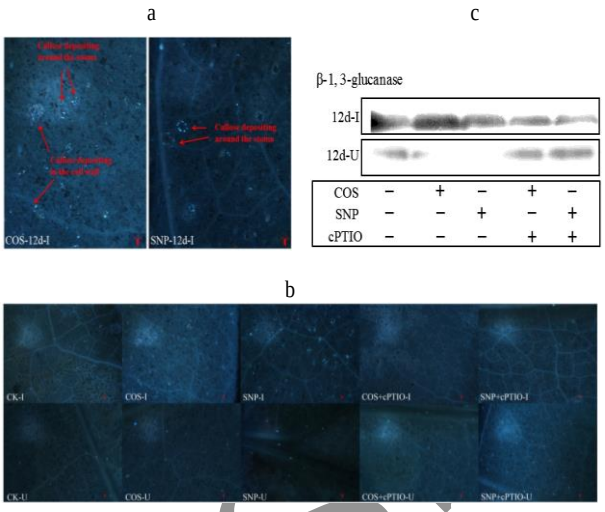

Fig. 3: Callose staining by aniline blue and β-1, 3-glucanase expression of different groups at 12 d in tobacco inoculated with TMV. (a) Fluorescence image of callose deposition in the cell wall and around the stoma in tobacco leaves induced by COS and SNP, respectively. The bright spots indicated by red arrow represent callose. (b) Callose deposition in differently treated tobacco leaves. Callose deposition was only observed in COS and SNP treated samples in the inoculated TMV leaves (I). (c) β-1, 3-glucanase expression in tobacco leaves with TMV infection at 12 d. I: inoculated TMV leaves; U: uninoculated TMV leaves

So we verified the calreticulin expression in a healthy plant at 1 d, and the result showed that COS and SNP treatment led to less calreticulin expression, while cPTIO also reversed this process. The result was opposite to that observed in tobacco leaves with TMV infection, suggesting the trend of COS-regulated calreticulin expression depends on whether the leaves are inoculated with TMV or not. Importantly, in either case, COS-regulated calreticulin expression was mediated by NO.

Discussion

COS can trigger innate immunity in various species of plants. To examine its antivirus mechanism, the early signal NO was studied in the current research. Increasing number of reports show that NO has a major role in plant and pathogen interaction (Klessig *et al.*, 2000; Romero-Puertas *et al.*, 2004; Fu *et al.*, 2010). However, the mechanism of NO-regulated TMV movement has rarely reported.

In the present work, NO generation in tobacco leaves with or without TMV infection was measured by Griess reagent. Results indicate that COS and SNP treatment could induce NO production under no TMV infection, but no significant difference in the NO content of experimental groups (control, COS± cPTIO and SNP ± cPTIO) was observed after TMV infection.

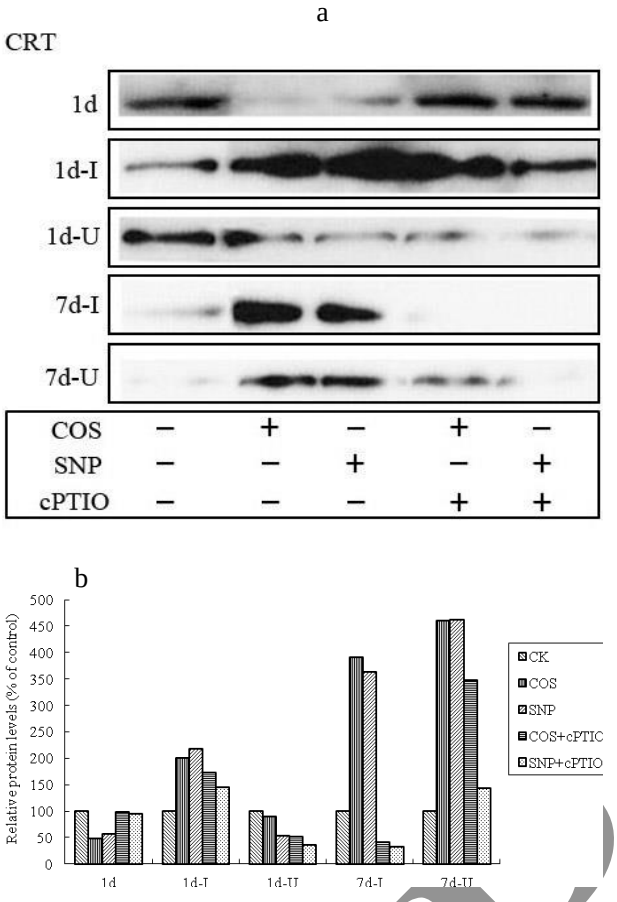

Fig. 4: Calreticulin expression in tobacco leaves with and without TMV infection. (a) 1 d: calreticulin expression in tobacco leaves at 1 d after different treatment in healthy tobacco plant; 1 d-I, 1 d-U, 7 d-I, 7 d-U: calreticulin expression in inoculated TMV leaves and uninoculated TMV leaves after TMV infection at 1 d and 7d in different samples. (b) Quantitative estimation of the result obtained in (a). Calreticulin, CRT

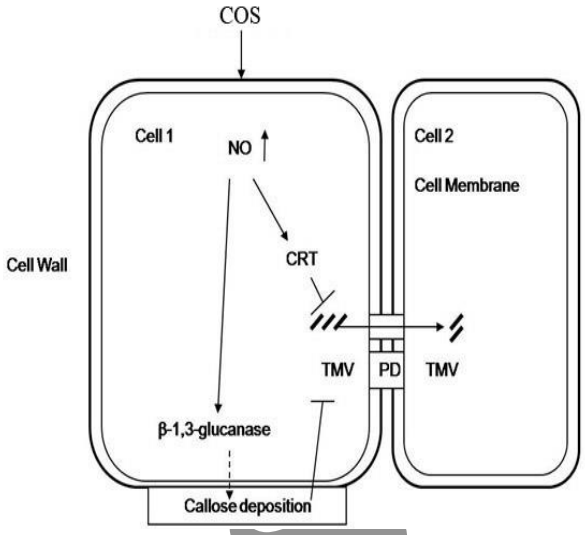

Fig. 5: Signaling pathways in COS-induced resistance to TMV in tobacco. Cells treated with COS lead to NO generation. NO may regulate β-1,3-glucanase and calreticulin (CRT) expression, which in turn resultes in callose deposition and restriction of TMV movement protein at plasmodesmata (PD). Finally, TMV movement through the plasmodesmata to adjacent cells is inhibited

The results primarily indicate that TMV infection may be more effective than COS on NO generation in this condition (Fu *et al*., 2010), and secondly suggested that, after signal transduction of NO, the extra NO maybe removed or stored in vivo in another form such as S-nitrosothils. The S-nitrosothils was further investigated, and results show that the S-nitrosothils contents in different experimental groups were at the same whether or not with TMV infection, suggesting S-nitrosothils may be not involved in COS-induced defense to TMV. While the NO and S-nitrosothils contents were obviously higher in the TMV inoculated leaves than that in the healthy tobacco leaves, indicating NO and S-nitrosothils were involved in the plant-viral interaction. Detection of S-nitrosylated proteins though biotin-switch assay may be a way to determine whether S-nitrosothiols is involved in COS-induced NO signal pathway in tobacco inoculated with TMV (Hu *et al.*, 2015). Finally, the expression of TMV-CP was measured to verify if NO had a role to play in COS-induced inhibition of TMV multiplication and movement. Results show that clearing NO led to increase expression of TMV-CP in the tobacco leaves, clearly indicating the role of NO in COS-induced resistance to TMV multiplication and movement.

Successful infection of the plant by a virus is determined by two possible processes: its movement from cell-to-cell through plasmodesmata and its rapid pass through the plant phloem (Carrington *et al.*, 1996). So the callose deposition, β-1, 3-glucanase and calreticulin protein expression were measured. Our results found that COS can induce callose deposition in the cell walls of TMV infected leaves, and cPTIO inhibits the function of COS. Moreover, COS-induced β-1, 3-glucanase expression is up-regulated in the TMV inoculated leaves, but down-regulated in uninoculated TMV leaves, both of which processes are controlled by NO. The results suggest that NO participates in regulating β-1, 3-glucanase expression and callose deposition. The results of our study are opposite to ever reported (Iglesias and Meins, 2000), suggesting there may be a distinct mechanism in COS-induced callose deposition and β-1, 3-glucanase expression. The mechanism of NO-mediated regulation of β-1, 3-glucanase expression is the next question leaving us to investigate.

Further, our results prove that COS-induced calreticulin overexpression in TMV infected leaves is

regulated by NO. It has been reported that the over-expression of calreticulin leads to inhibition of TMV movement (Chen *et al.*, 2005), and our results indicate that NO may regulate calreticulin expression to inhibit TMV movement in COS-induced defense response to TMV. In animal immune system, calreticulin interacts with the endothelium to stimulate the release of NO (Bansal *et al.*, 2008), and in turn NO affects calreticulin expression (Kopecka *et al.*, 2011). However, in plant cells, the interaction between NO and calreticulin was not studied and reported so far. Our finding indicates that in plants there may exist similar interaction between NO and calreticulin as in the animals; and those work will aid in uncovering the mechanisms of COS-induced defense response to TMV. The major challenge ahead is to study how NO regulates calreticulin expression.

Conclusion

In this work, we investigated the role of NO in COS-elicited defense response to TMV in *Nicotiana tabacum* cv. Huangmiaoyu NN, and described the corresponding signaling network (Fig. 5). According to our discovery, we can postulate that treatment of cells to COS leads to NO generation, which in turn regulates β-1, 3-glucanase expression that may be related with callose deposition at plasmodesmata, and that NO could regulate calreticulin expression, and finally, that TMV movement through the plasmodesmata to adjacent cells could be inhibited. This is the first study on plant-virus interaction to report the relationship among NO, callose deposition, β-1, 3-glucanase, and calreticulin. The findings might be an important step for amplification of NO and COS signaling in plant defense response.

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Reference

Bansal, S., M. Gaspari, H.G. Raj, A. Kumar, G. Cuda, E. Verheij, Y.K. Tyagi, P. Ponnan, R.C. Rastogi and V.S. Parmar, 2008. Calreticulin transacetylase mediates the acetylation of nitric oxide synthase by polyphenolic acetate. *Appl. Biochem. Biotechnol.*, 144: 7–45

Beffa, R.S. and F.Jr. Meins, 1996. Pathogenesis-related functions of plant β-1,3-glucanases investigated by antisense tranformation-a review. *Gene*, 179: 97–103

Bucher, G.L., C. Tarina, M. Heinlein, F.D. Serio, Jr. F. Meins and V.A. Iglesias, 2001. Local expression of β-1,3-glucanase enhances symptoms of TMV infection in tobacco. *Plant J.*, 28: 361–369

Carrington, J.C., K.D. Kasschau, S.K. Mahajan and M.C. Schaad, 1996. Cell-to-cell and long distance transport of virus in plants. *Plant Cell*, 8: 1669–1681

Chandra, S., N. Chakraborty, K. Panda and K. Acharya, 2017. Chitosan-induced immunity in Camellia sinensis (L.) O. Kuntze against blister blight disease is mediated by nitric-oxid. *Plant Physiol. Biochem.*, 115: 298–307

Chen, M.H., G.W. Tian, Y. Gafni and V. Citovsky, 2005. Effects of calreticulin on viral cell-to-cell movement. *Plant Physiol.*, 138: 1866–1876

Davydova, V.N., V.P. Nagorskaya, V.I. Gorbach, A.A. Kalitnik, A.V. Reunov, T.F. Solov'eva and I.M. Ermak, 2011. Chitosan antiviral activity: dependence on structure and depolymerization method. *Prikl. Biokhim. Mikrobiol.*, 47: 13–118

Del Bem, L.E.V., 2011. The evolutionary history of calreticulin and calnexin genes in green plants. *Genetica*, 139: 255–259

Delledonne, M., Y.J. Xia, R.A. Dixon and C. Lamb, 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature*, 394: 5–588

Delp, G. and E.T. Palva, 1999. A novel flower-specific Arabidopsis gene related to both pathogen-induced and developmentally regulated plant beta-1, 3-glucanase genes. *Plant Mol. Biol.*, 39: 565–575

Deng, L.L., Y.H. Zhou and K.F. Zeng, 2015. Pre-harvest spray of oligochitosan induced the resistance of harvested navel oranges to anthracnose during ambient temperature storage. *Crop Prot.*, 70: 70–76

Ellinger, D. and C.A. Voigt, 2014. Callose biosynthesis in arabidopsis with a focus on pathogen response: what we have learned within the last decade. *Ann. Bot-London*, 114: 1349–1358

Fu, L.J., K. Shi, M. Gu, Y.H. Zhou, D.K. Dong, W.S. Liang, F.M. Song and J.Q. Yu, 2010. Systemic Induction and Role of Mitochondrial Alternative Oxidase and Nitric Oxide in a Compatible Tomato-Tobacco mosaic virus Interaction. *Mol. Plant Microbe. Int.*, 23: 39–48

Hassan, A.M., C. Wesson and W.R. Trumble, 1995. Calreticulin is the major ca^{2+} storage protein in the endoplasmic-reticulum of the pea plant (*Pisum sativum*). *Biochem. Biophy. Res. Co.*, 211: 54–59

Hong, J.K., B.W. Yun, J.G. Kang, M.U. Raja, E. Kwon, K. Sorhagen, C.C. Chu, Y.Q. Wang and G.J. Loake, 2008. nitric oxide function and signalling in plant disease resistance. *J. Exp. Bot.*, 59: 147–154

Hu, J.L., X.H. Huang, L.C. Chen, X.W. Sun, C.M. Lu, L.X. Zhang, Y.C. Wang and J.R. Zuo, 2015. Site-specific nitrosoproteomic identification of endogenously S-nitrosylated proteins in Arabidopsis. *Plant Physiol.*, 167: 1731–1746

Hu, Y., J. Cai, Y.M. Du, J.G. Lin, C.G. Wang and K.J. Xiong, 2009. Preparation and anti-TMVactivity of guanidinylated chitosan hydrochloride. *J. Appl. Polym. Sci.*, 112: 3522–3528

Iglesias, V.A. and Jr. F. Meins, 2000. Movement of plant viruses is delayed in a β-1,3-glucanase-deficient mutant showing a reduced plasmodesmatal size exclusion limit and enhanced callose deposition. *Plant J.*, 21: 157–166

Iriti, M. and F. Faoro, 2008. Abscisic acid is involved in chitosan-induced resistance to tobacco necrosis virus (TNV). *Plant Physiol. Biochem.*, 46: 1106–1111

Iriti, M., M. Sironi, S. Gomarasca, A.P. Casazza, C. Soave and F. Faoro, 2006. Cell death-mediated antiviral effect of chitosan in tobacco. *Plant Physiol. Biochem.*, 44: 893–900

Jia, X.Y., L.H. He, R.L. Jing and R.Z. Li, 2009. Calreticulin: conserved protein and diverse functions in plants. *Physiol. Plant.*, 136: 127–138

Klessig, D.F., J. Durner, R. Noad, D.A. Navarre, D. Wendehenne, D. Kumar, J.M. Zhou, J. Shah, S.Q. Zhang, P. Kachroo, Y. Trifa, D. Pontier, E. Lam and H. Silva, 2000. Nitric oxide and salicylic acid signaling in plant defense. *P. Natl. Acad. Sci. USA*, 97: 8849–8855

Kochkina, Z.M. and S.N. Chirkov, 2001. Influence of the Chitosan Oligomer on the Phage Particles and Reproduction of Phage 1-97A in the Culture of Bacillus thuringiensis. *Microbiology*, 70: 706–710

Kopecka, J., I. Campia, D. Brusa, S. Doublier, L. Matera, D. Ghigo, A. Bosia and C. Riqanti, 2011. Nitric oxide and P-glycoprotein modulate the phagocytosis of colon cancer cells. *J. Cell Mol. Med.*, 15: 1492–1504

Kulikov, S.N., S.N. Chirkov, A.V. Il'ina, S.A. Lopatin and V.P. Varlamov, 2006. Effect of the molecular weight of chitosan on its antiviral activity in plants. *Appl. Biochem. Microbiol.*, 42: 200–203

Laporte, C., G. Vetter, A.M. Loudes, D.G. Robinson, S. Hillmer, C. Stussi-Garaud and C. Ritzenthaler, 2003. Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of Grapevine fanleaf virus movement protein in tobacco BY-2 cells. *Plant Cell*, 15: 2058–2075

Li, P.Q., J.L. Robert and Z.M. Cao, 2016. Structural characterization of oligochitosan elicitor from *Fusarium sambucinum* and its elicitation of defensive responses in *Zanthoxylum bungeanum*. *Int. J. Mol. Sci.*, 17: 2076

Li, W.L., Y.S. Zhao, C.J. Liu, G.B. Yao, S.S. Wu, C.Y. Hou, M.C. Zhang and D.M. Wang, 2012. Callose deposition at plasmodesmata is a critical factor in restricting the cell-to-cell movement of Soybean mosaic virus. *Plant Cel. Rep.*, 31: 905–916

Lindermayr, C., G. Saalbath and J. Durner, 2005. Protemic identification of S-Nitrosylated proteins in Arabidopsis. *Plant Physiol.*, 137: 921–930

Lu, H., X.M. Zhao, W.X. Wang, H. Yin, J.G. Xu, X.F. Bai and Y.G. Guang, 2010. Inhibition effect on tobacco mosaic virus and regulation effect on calreticulin of oligochitosan in tobacco by induced $Ca^{(2+)}$ influx. *Carbohyd. Polyme.*, 82: 136–142

Ma, Z.X., L.Y. Yang, H.X. Yan, F.J. Kennedy and X. Meng, 2013. Chitosan and oligochitosan enhance the resistance of peach fruit to brown rot. *Carbohyd. Polym.*, 94: 272–277

Rabea, E.I., M.E. Badawy, C.V. Stevens, G. Smagghe and W. Steurbaut, 2003. Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules*, 4: 457–1465

Romero-Puertas, M.C., M. Perazzolli, E.D. Zago and M. Delledonne, 2004. Nitric oxide signalling functions in plant-pathogen interactions. *Cell. Microbiol.*, 3: 795–803

Shang, W.J., Y.F. Wu, H.S. Shang, X.M. Zhao and Y.G. Du, 2008. The inactivating effect of chito-oligosaccharides on TMV particles in vitro. *Bing Du Xue Bao*, 24: 76–78

Shinshi, H., H. Wenzler, J.M. Neuhaus, G. Felix, J. Hofsteenge and F. Meins, 1988. Evidence for N-terminal and C-terminal processing of a plant defense-related enzyme-primary structure of tobacco prepro-beta-1, 3-glucanase. *P. Natl. Acad. Sci. USA.*, 85: 5541–5545

Tada, Y., S.H. Spoel, K. Pajerowska-Mukhtar, Z.L. Mou, J.Q. Song, C. Wang, J.R. Zuo and X.N. Dong, 2008. Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science*, 321: 952–956

Ton, J. and B. Mauch-Mani, 2004. Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *Plant J.*, 38: 119–130

Wendehenne, D. and J.T. Hancock, 2011. New frontiers in nitric oxide biology in plant preface. *Plant Sic.*, 181: 507–508

Yin, H., X.M. Zhao and Y.G. Du, 2011. Oligochitosan: A plant diseases vaccine-A review. *Carbohyd. Polym.*, 82: 1–8

Yun, B.W., M.J. Skelly, M. Yin, M. Yu, B.G. Mun, S.U. Lee, A. Hussain, S.H. Spoel and G.J. Loake, 2016. Nitric oxide and S-nitrosoglutathione function additively during plant immunity. *New Phytol.*, 211: 516–526

Zhang, H.Y., X.M. Zhao, J.L. Yang, H. Yin, W.X., Wang, H. Lu and Y.G. Du, 2011. Nitric oxide production and its functional link with OIPK in tobacco defense response elicited by chitooligosaccharide. *Plant Cell Rep.*, 30: 1153–1162

Zhao, X.M., X.P. She, Y.G. Du and X.M. Liang, 2007. Induction of antiviral resistance and stimulary effect by oligochitosan in tobacco. *Pestic. Biochem. Phys.*, 87: 78–84

Zhou, J., F.F. Jia, S.J. Shao, H. Zhang, G.P. Li, X.J. Xia, Y.H. Zhou, J.Q. Yu and K. Shi, 2015. Involvement of nitric oxide in the jasmonate-dependent basal defense against root-knot nematode in tomato plants. *Front. Plant Sci.*, 6: 193

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