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β-1,3-Glucan with different degree of polymerization induced different defense responses in tobacco

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ABSTRACT

In order to elucidate defense-inducing activities in tobacco, curdlan oligosaccharides (CRDO) with low degree of polymerization (DP) were prepared for comparison with high DP Laminaran (Lam). Fast response defenses such as extracellular pH shift, hydrogen peroxide (H_2O_2) production, nitric oxide (NO) release and stomatal movement under light as well as the protection against tobacco mosaic virus (TMV) were performed. CRDO triggered faster extracellular alkalinization than Lam. Refractory states analysis showed there were different perception modes between β-1,3-glucan and chitosan oligosaccharides (COS). Elicitors induced cells to generate NO probably via the nitric oxide synthase (NOS) pathway. Moreover, CRDO exhibited more intensive in stomatal movement than Lam. However, protection against TMV implied Lam played a longer term role in defense responses than CRDO. Therefore, we concluded β-1,3-glucan with low DP played a vital role in the rapid responses, whereas high DP β-1,3-glucan was responsible for the longer term effects.

1. Introduction

Microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) can induce plant innate immunity to protect plants from microbial pathogens (Jaulneau et al., 2010; Jones & Dangl, 2006). Some of elicitors have been shown to be carbohydrates and can be divided into two kinds according to their origins. Endogenous elicitors are oligogalacturonans mostly derived from pectins, or xyloglucans, or cellodextrins from cellulose or hemicellulose (Aziz et al., 2007; Paulert, Ebbinghaus, Urlass, & Moerschbacher, 2010). The other is exogenous carbohydrate elicitors, including the chitosan from fungal cell walls or exoskeleton of arthropod (Li et al., 2009; Yin, Zhao, & Du, 2010) and β-glucan from *Phytophythora sojae*, *Pyricularia oryzae* and *Laminaria digitata* (Klarzynski et al., 2000; Sharp, McNeil, & Albersheim, 1984).

Exogenous carbohydrate oligosaccharides are also termed one kind of oligosaccharins (Darvill et al., 1992). Oligosaccharins as elicitors have enlivened the public's hopes of a green bio-pesticide. Some of them have impacts on plant growth and development as nontraditional plant hormones, while others are regarded as elicitors to trigger plant resistance against diseases (Darvill et al., 1992; Liu, Cheng, Liu, Du, & Bai, 2008).

Laminaran (Lam), which derived from *L. digitata*, is a β-1,3-glucan. The degree of polymerization (DP) is 25–33 and there are three single β-glucose branches randomly substituted at position 6 (Read, Currie, & Bacic, 1996). Lam has been shown to induce various defense and resistance reactions in many plants, such as alfalfa (Kobayashi, Tai, Kanzaki, & Kawazu, 1993), rice (Inui, Yamaguchi, & Hirano, 1997), tobacco (Klarzynski et al., 2000), grapevine (Aziz et al., 2003) and *Arabidopsis* (Menard et al., 2004). According to Menard et al. (2004) Lam induced the ethylene-dependent pathogenesis-related (PR) proteins, whereas chemically sulfated Lam with a degree of sulfation of 2.4 triggered the expression of ethylene- as well as salicylic acid-dependent PR proteins. However, laminaran from different areas and genus varied considerably in structure and content (Chizhov et al., 1998; Zvyagintseva et al., 1999). Compared with the 10% of the dry weight of *L. digitata* in Europe described above, laminaran extracted from *L. japonica*, the most widely distributed brown algae in China, only accounts for 0.5–1% (Jin et al., 2009; Klarzynski et al., 2000; Zyyagintseva et al., 2003). Therefore, it is difficult to use this reagent widely in China. High cost of imported Lam from Europe as well as low content in Chinese brown algae make it urgent to find another cheap and abundant β-1,3-glucan resource to replace Lam. Here, curdlan was hydrolyzed into curdlan oligosaccharides (CRDO) by acid and compared with Lam. Curdlan, a β-(1→3) linked D-glucose polysaccharide but without side chains, was first detected in *Alcaligenes faecalis* var. *myxogenes* and belongs to microbial exopolysaccha-

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rides and has been manufactured industrially (Harada, Masada, Fujimori, & Maeda, 1966; Sutherland, 1999). Curdlan is insoluble in alcohols and most organic solvents, but dissolves in dilute basic and dimethyl sulfoxide (DMSO) (McIntosh, Stone, & Stanisich, 2005). Another reason why we used CRDO instead of Lam oligosaccharides was that the degradation products of Lam may contain some oligosaccharides with β-1,6-glucosidic bond, which could not elucidate the biological activities of β-1,3-linked glucosyl residues clearly. Plentiful resources of curdlan would probably allow the use of β-1,3-glucan to be widely used in plant treatment.

Using structural analysis of the β-1,3-glucan from different sources by matrix assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF-MS) and fast atom bombardment mass spectrometry (FAB-MS), it was shown that there were diverse DP distributions in different β-1,3-glucan and our belief is that the disease resistance effects, to some extent, depended on the DP of the oligosaccharides. For example, Aziz et al. (2007) demonstrated that DP was of vital importance for the defense response induced by cellodextrins (β-1,4-glucoside residues) in grapevines. In this paper, the DP of Lam and that of CRDO were detected and the different defense effects induced by these two kinds of β-1,3-glucan were investigated in tobacco.

Chitosan oligosaccharides (COS), built up of β-1,4-glucosamines, have been considered to be a potent plant elicitor in various plants such as tobacco, rapeseed, strawberry, etc. (Eikemo, Stensvand, & Tronsmo, 2003; Yin et al., 2010). COS with a similar DP to CRDO, prepared by enzymatically degrading chitosan with a membrane separation coupling technique was used as a control.

In this paper, the effects of high DP β-1,3-glucan (Lam), low DP β-1,3-glucan (CRDO) and similar low DP COS were investigated in tobacco. Extracellular pH shift, H_2O_2 production, NO release of tobacco suspension cells as well as stomatal movement under light was detected. Furthermore, the protection effects of these elicitors against tobacco mosaic virus (TMV) in tobacco plant were examined.

2. Materials and methods

2.1. Chemicals

Laminaran was purchased from Sigma. Sodium nitroprusside (SNP), 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), 3-amino,4-aminomethyl-2′,7′-difluorescein diacetate (DAF-FM DA) and L-nitro-arginine methyl ester (L-NAME) were products of Beyotime Institute of Biotechnology. 2′,7′-Dichlorofluorescin diacetate (H_2DCF-DA) was purchased from Biotium. All of them were analytical reagents.

2.2. Preparation of CRDO and COS

Curdlan (5 g) was dissolved in 600 ml DMSO and lithium chloride (LiCl) and then stirred overnight to ensure full dissolution. Acid was slowly added into the curdlan solution at 105 °C for 150 min. After the solution was cooled down to room temperature, the resulting solution was neutralized by NaOH and then dialyzed against distilled water using a regenerated cellulose membrane with a cutoff of 200 Da to remove DMSO and LiCl. The dialysed solution was lyophilized to yield CRDO.

COS was produced by enzymatic hydrolysis of chitosan (the degree of deacetylation is more than 95%) (Yin, Du, & Zhang, 2009). Chitosan (0.5 g) was dissolved in 10 ml of 2% glacial acetic acid solution under strong agitation (600 rpm). The pH value was adjusted to 5.6 after 15 min of solution stabilization. Enzyme was dissolved in 0.05 M acetate buffer at 40 °C. The mixture was heated at 95 °C for 10 min to deactivate the enzyme. And was filtered through a hollow-fiber membrane to separate the supernatants and lyophilized.

2.3. MALDI-TOF-MS detection

MALDI-TOF-MS was performed on a Burker Autoflex time-of-flight mass spectrometer (Bruker Daltonics, Germany) (Hu, Jiang, Xu, Pan, & Zou, 2006). The instrument harbored a pulsed nitrogen laser operated at 337 nm and accelerating potential in the range of ±20 kV with a delayed ion-extraction device. Samples were added on a steel plate with 384 spots. The laser energy was regulated above the threshold for good resolution and signal-to-noise ratio (S/N). Mass spectrum was obtained in the positive ion linear mode with delayed extraction for 100 ns and typically added up to 50 laser shots. A matrix of 2,5-dihydroxybenzonic acid (2,5-DHBA) and each elicitor were mixed at 1:1 (v/v), and 10 mM NaCl was added to the solution. The mass calibration procedure was employed prior to the analysis. Deionized water used for all experiments was purified with a WaterPro PS water system (Labconco, USA).

2.4. Plant material

2.4.1. Cell-suspension culture

Tobacco (*Nicotiana tabacum* var. Samsun NN) cell-suspension cultures were maintained in 100 mL Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid and sucrose under stirring (120 rpm) at 25 °C with a photoperiod of 12 h on a 7 days growth cycle (10% v/v inoculum). The cells were used to do experiments following harvest and re-suspension in fresh culture medium for 24 h. All the processes were under aseptic conditions.

2.4.2. Plant material

Tobacco (*N. tabacum* var. Samsun NN) was grown in a greenhouse at 80% humidity, with a photoperiod of 16 h light at 24 °C and 8 h dark at 22 °C, and 6-leaf-stage plants were used for the experiments.

2.5. Detection of extracellular medium pH value

The pH values were measured with Jenway 4330 conductivity and pH meter (Jenway, England) during the continuous shaking of cells at room temperature. The solution of CRDO, Lam and COS (500 μg/mL) and water were added into the medium, respectively. After the detection of CRDO treatment, the pH meter was washed twice and dried for Lam 1 min later and elicitors were dissolved in water and added again at the same concentrations 1 h later.

2.6. H_2O_2 burst measurement in tobacco cell cultures

100 μL of cells were placed in a 96 well cell culture plate (Greiner, Germany). H_2O_2 burst was measured in tobacco suspension cells loaded with H_2DCF-DA (1 μM) using Spectra MAX Gemini EM (Molecular Devices, USA). Elicitors (500 μg/mL) were added into wells prior to detection. The relative fluorescence was read from the bottom of the plates with excitation at 488 nm and emission at 530 nm. The software Soft Max® Pro 5 was used to analyze the results. The relative fluorescence unit was equal to the amount of cells loaded with probes minus those without probes.

2.7. NO burst measurement in tobacco cell cultures

Nitric oxide (NO) measurement was performed using the fluorescent probe DAF-FM DA (2.5 μM) with a modification of the method described by Zhao, She, Du, and Liang (2007). After cells were loaded with inhibitors at final concentration of cPTIO

(500 μM), L-NAME (10 mM) and NaN_3 (5 mM) for 30 min, respectively, cells were washed twice to get rid of the interference, followed by the incubation with DAF-FM DA for 30 min. An identical washing method was applied to cells again. After 10 min accommodation, SNP (NO donor), CRDO, Lam and COS, respectively, were added into cells at final concentration of SNP (1 mM) and three elicitors (500 μg/mL). Results of the NO production from tobacco cells were observed after 5 min by inverted fluorescence microscope equipped with Canon camera.

2.8. Stomatal movement analysis under light

Leaves from tobacco plant were harvested at the 6-leaf-stage and epidermal peels were prepared as previously described (Allegre et al., 2007, 2009). After the mesophyll was removed by forceps carefully, the epidermal peels were washed and placed in a 6 well cell culture plate (Costar®, Corning, USA) containing an equilibration buffer (10 mM Mes–KOH, pH 6.5) for 30 min at 24 °C in darkness. In order to check the inhibition of stomatal opening under light, epidermal peels were added elicitors in buffer (50 mM KCl, 10 mM Mes–KOH, pH 6.5), followed by illumination for 3 h at 24 °C at 1500 lx and fixation in ethanol. Stomatal apertures under light were calculated under a light microscope with a calibrated micrometer scale (Li et al., 2009). In the other experiment, epidermal peels were in buffer (30 mM KCl, 10 mM Mes–KOH, pH 6.5) at the same illumination condition. After 3 h to promote stomatal opening, elicitors (500 μg/mL) were added to induce the stomatal closure under light for 1 h. The fixation and calculation were as described above.

2.9. Protection assays of elicitors in tobacco plant against TMV

All leaves of tobacco were sprayed with 50, 200 and 500 μg/mL of CRDO and Lam and 50 μg/mL of COS and water as control, respectively. 24 h later, plants were inoculated mechanically with TMV solution. TMV was conserved in our laboratory and harvested the supernatant of extraction according to Lu et al.'s (2010) method. Quantification of diseases development in tobacco leaves was measured at 3 days after treatment.

2.10. Statistical analysis

Statistical analyses were carried out by SPSS 13.0 package (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± S.D. One-way ANOVA and Student's *t*-test followed by a Bonferroni correction were performed to compare the different groups. Values of $p < 0.05$ were considered to determine statistical significance.

3. Results

3.1. Structural analyses of three elicitors

MALDI-TOF-MS has been proved to be a powerful tool for defining the DP profiles and molecular mass in complex mixtures of polysaccharide. The mass spectrum showed that peaks were corresponding to the mass number of $(M + Na)^+$. In Fig. 1A, CRDO was composed mainly of DP 2–10 with the maximum of DP 4 and 5. The Lam accounted for at least DP 40 with the abundance peaking at DP 25–26 (m/z) as well as the low DP mixture (Fig. 1B). The DP of COS was ranging from 3 to 10, mainly DP 3–6, with the highest content of DP 4 (Fig. 1C). There were 16 mass units in Lam between G-chains (mannitol-free) and M-chains (mannitol-containing) oligosaccharides while the "minus 18" signals maybe stand for the cyclisation, which closely resemble the data of laminaran from *Cystoseira crinita* (Chizhov et al., 1998).

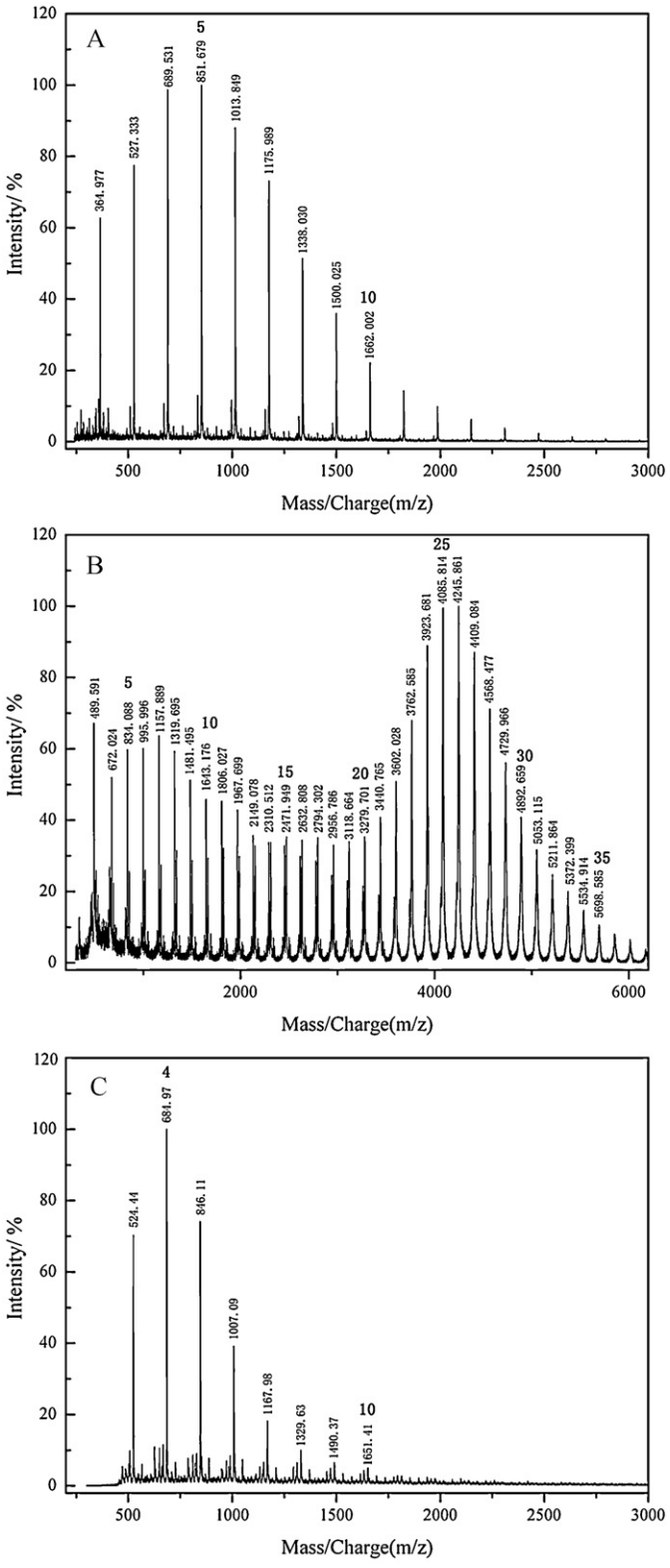

Fig. 1. MALDI-TOF mass spectra of elicitors. The mass charge (*m*/*z*) values were accounted for the nominal masses of the psedomolecular ions $[M + Na]^+$. (A) CRDO, (B) Lam, (C) COS.

3.2. Elicitors triggered rapid and strong extracellular alkalinization response in tobacco

As one of rapid responses, alkalinization of the extracellular medium induced by elicitors has been proven to be in response

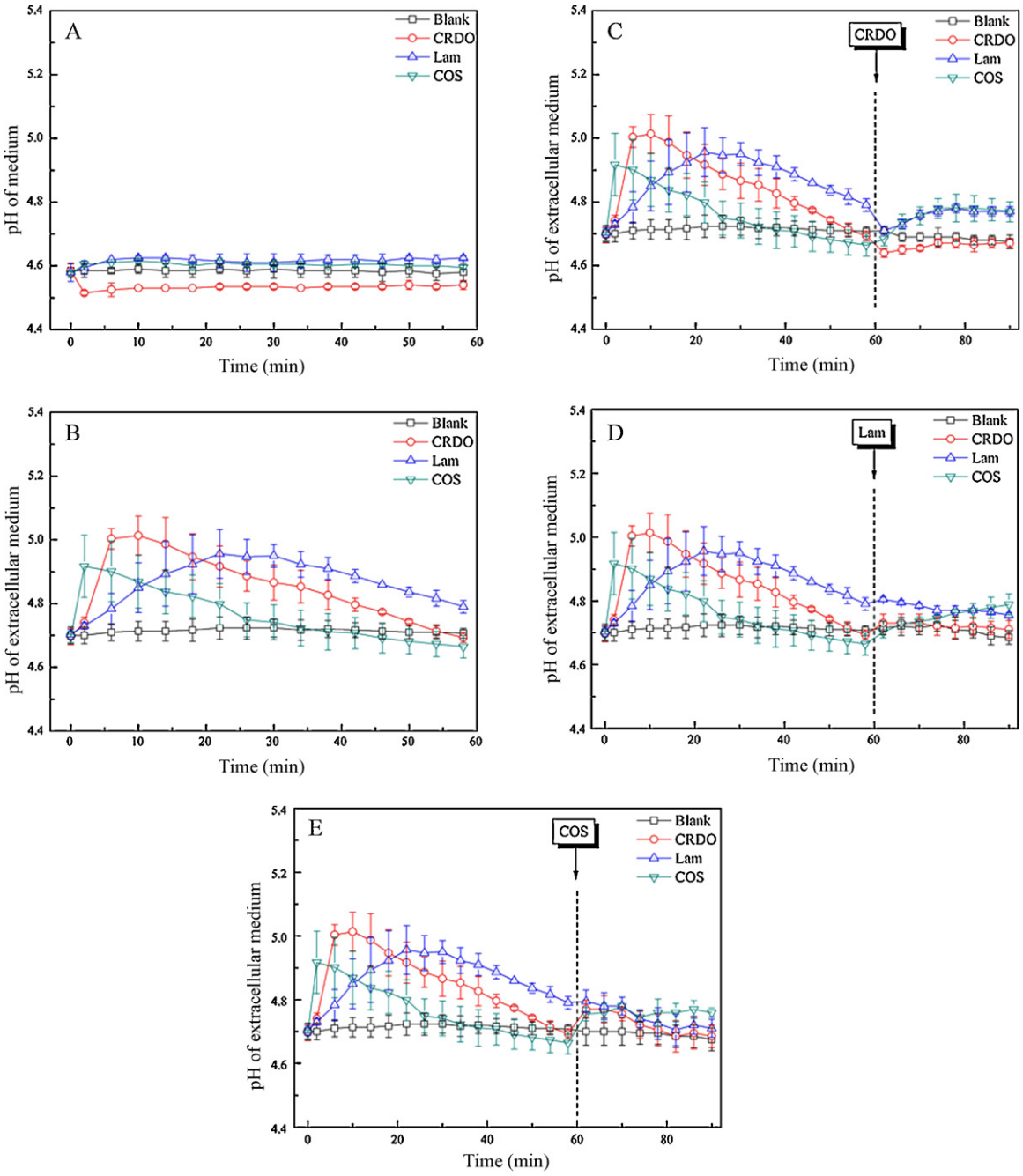

Fig. 2. Alkalinization of the extracellular medium of tobacco suspension cells responded to incubation with three elicitors. (A) The pH values of elicitors in the culture medium without cells. (B) Time courses of pH changed after treated with CRDO (500 μg/mL, ○), Lam (500 μg/mL, △), COS (500 μg/mL, ▽) and controls with water (□). (C) Refractory state analysis with CRDO. Elicitors at the same concentration were added again 1 h later as other refractory state analysis. (D) Refractory state analysis with Lam. (E) Refractory state analysis with COS.

to various elicitors (Klarzynski et al., 2000). As shown in Fig. 2A, pH values were measured after three elicitors were added into cell-free culture medium. COS solution and Lam solution were slight alkaline, while CRDO solution was acidic. Tobacco suspension cells had rapid and strong alkalinization of extracellular medium as soon as the addition of these three elicitors with different patterns (Fig. 2B). CRDO caused pH-ascended quickly and reached the maximum pH shift at 10 min, followed by a gradual re-acidification of the medium. At early stages, pH shift induced by CRDO treatment was quicker than by Lam treatment, but became lower and finally backed to its origin level at the end of the experiment.

To investigate whether the presence of distinct perception systems for β-1,3-glucan and COS, elicitor was added to the cells again. It should be noted that tobacco cells pretreated with CRDO or Lam, respectively, were shown to be refractory to a second elicitation of CRDO or Lam (Fig. 2C and D). It was assumed that β-1,3-glucan, regardless of DP, would not lead to alkalinization of extracellular medium pretreated with β-1,3-glucan again, while pH of COS pretreatment would increase again under the second stimulation of CRDO or Lam. Furthermore, pH shift elicited by CRDO treatment was also faster than Lam treatment (Fig. 2C and D). When COS was added again 1 h later into the culture medium initially stimulated by CRDO, Lam and COS, respectively, the suspension cells

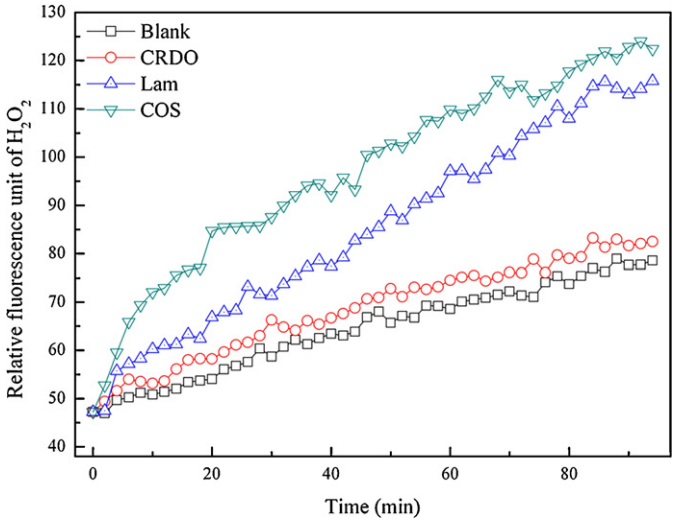

Fig. 3. Time courses of the relative fluorescence unit of H_2O_2 burst. The solution of CRDO (500 μg/mL, ○), Lam (500 μg/mL, △), COS (500 μg/mL, ▽) and controls with water (□), respectively, were added into a 96 well cell culture plate with 100 μL tobacco suspension cells. Representative data from one of three replicates were showed.

with CRDO previously showed the similar quick and strong alkalinization as well as the gradual re-acidification (Fig. 2E).

3.3. H_2O_2 burst induced by elicitors in tobacco suspension cell

For each elicitor, the concentration of 500 μg/mL were found to be suitable for cell research in our laboratory (data not shown) and reported previously by a French research group (Aziz et al., 2007).

The H_2O_2 burst triggered by three elicitors is shown in Fig. 3. Tobacco suspension cells released the oxidative burst as soon as COS treatment. The amounts surged and accumulated with the time course. CRDO and Lam were able to induce the H_2O_2 release and played similar roles at the beginning within 5 min, which was easily neglected. Compared with the line-correlation-increasing of H_2O_2 release amount induced by Lam in the later stage, the amount of H_2O_2 induced by CRDO slightly promoted during the detection course.

3.4. NO burst induced by elicitors in tobacco suspension cells

To study the effects of three elicitors on the production of NO in tobacco suspension cells at early stage, the NO-sensitive fluorescent probe DAF-FM DA were used. NO production of tobacco cells were directly scrutinized by inverted fluorescence microscope. Three elicitors as well as the NO donor, SNP, enabled them to stimulate the NO generation (Fig. 4B–E). From Fig. 4F–J, the scavenger cPTIO had great impacts on the elimination of NO, even to the cells treated with SNP. The results of addition of L-NAME, selective inhibitor of NOS, were depicted in Fig. 4K–O. The effects of three elicitors triggering NO burst were sharply weak but still with faint fluorescence. On the other hand, NaN_3, one of NR inhibitor, almost had no effects on the inhibition of NO production, as shown in Fig. 4P–T.

As already stated above, it was assumed that CRDO, Lam and COS managed to induce NO release from tobacco suspension cells and were likely to share similar mechanism of NO production. NOS mainly took part in NO burst in tobacco treated with elicitors instead of NR.

3.5. Inhibition effects of elicitors on the stomatal opening and induction effects of elicitors on the stomatal closure under light

Epidermal cells are known as first obstacles to the biotic and abiotic stresses. The effects of CRDO, Lam and COS on stomatal movements in tobacco were investigated. Firstly, whether these elicitors have the ability to inhibit the stomatal opening was taken

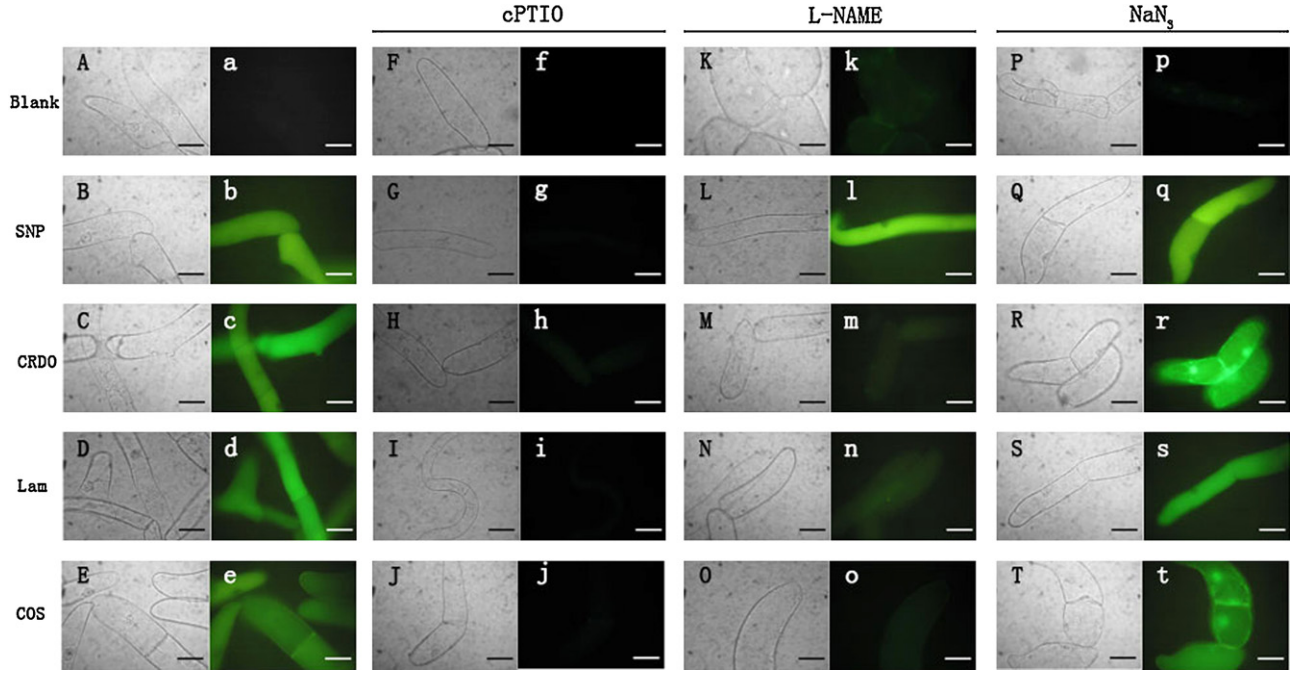

Fig. 4. Inverted fluorescence microscope of elicitors-induced production of NO in tobacco suspension cells loaded with DAF-FM DA (2.5 μM) before treatment with elicitors. Capital letters stood for bright field images and lower case letters represented fluorescent images. (A) Cells without any treatment. (B–E) Cells treated with SNP, CRDO, Lam and COS. (F) Cells treated with cPTIO before loaded with DAF-FM DA. (G–J) Cells with cPTIO were loaded with DAF-FM DA and treated with SNP, CRDO, Lam and COS, respectively. (K) Cells treated with L-NAME before loaded with DAF-FM DA. (L–O) Cells with L-NAME were loaded with DAF-FM DA and treated with SNP, CRDO, Lam and COS, respectively. (P) Cells treated with NaN_3 before loaded with DAF-FM DA. (Q–T) Cells with NaN_3 were loaded with DAF-FM DA and treated with SNP, CRDO, Lam and COS, respectively. Bars represented 50 μm.

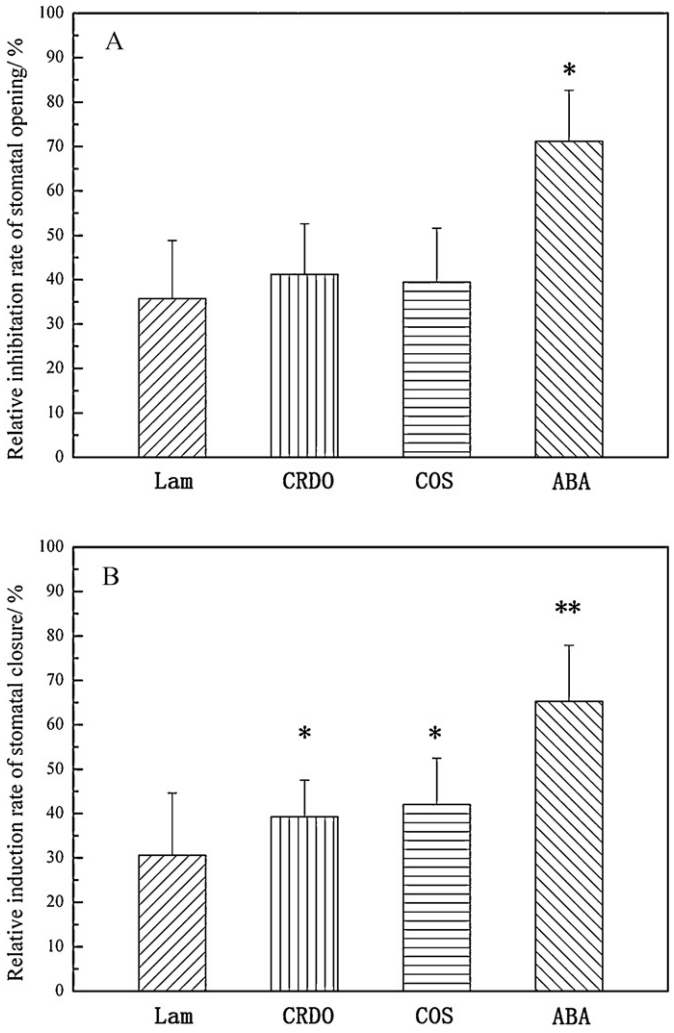

Fig. 5. Tobacco epidermal peels were incubated with Lam, COS, CRDO (both at the concentration of 500 μg/mL) and ABA (50 μM) on the inhibition of stomatal opening (A) and induction of stomatal closure under light (B), with water as a blank control. The relative inhibition and the induction rates, respectively, were calculated between the elicitors and control. The data represented the mean of 60 with three different epidermal peels. Values with asterisks were statistically different ($^{*}p < 0.05$, $^{**}p < 0.01$).

into account. To our expectation, abscisic acid (ABA), as a positive control, induced a significant reduction of stomatal aperture (68.75% compared with control). COS and CRDO with low DP showed similar relative inhibition rate of stomatal opening, but still far away from ABA, whereas the Lam induced a slight lower than the other elicitors on stomatal aperture (Fig. 5A). Mechanisms between stomatal opening and stomatal closure denoted a bifurcating signaling pathway (Mishra, Zhang, Deng, Zhao, & Wang, 2006). Secondly, we performed elicitors to induce stomatal closure under light. Elicitor-induced stomatal closure and the inhibition of stomatal opening shared a similar situation in this study. ABA has most significant effect on inhibition of stomatal opening and induction of closure, followed by CRDO and COS. Lam had least effect (Fig. 5B). Two oligosaccharides elicitors, CRDO and COS with similar DP, exhibited similar effects on the movement of stomata.

3.6. Protection assays of elicitors on tobacco against TMV

To investigate the effects of elicitors in tobacco plant, three elicitors were compared at different concentrations. The inhibition rate and the concentration of CRDO had dose dependent manners. CRDO

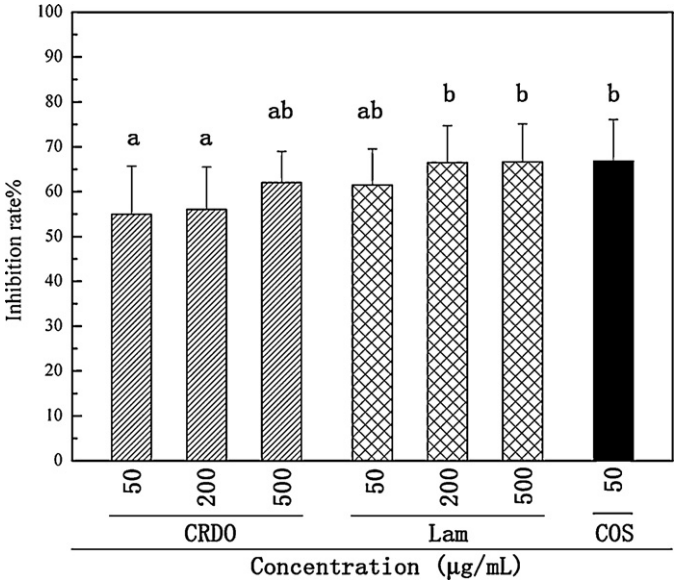

Fig. 6. Effects of elicitors protected tobacco leaves against TMV. Tobacco plants were pre-treated with different concentrations of CRDO, Lam and COS. The "a" and "b" stood for values significant difference ($p < 0.05$).

exhibited the best inhibition against TMV at 500 μg/mL, while it was similar to Lam at low dose (50 μg/mL). 200 μg/mL of Lam had greatly impacts on TMV inhibition, while the higher concentrations (500 μg/mL) would not lead to better effects. The inhibition rates were 66.48% and 66.66%, respectively. 50 μg/mL of COS was used as a positive control according our former results (Zhao, She, Du, et al., 2007) and the inhibition rate was 66.84%, which had similar results to that of Lam at 200 or 500 μg/mL, definitely surpassed CRDO with similar DP (Fig. 6).

4. Discussion

In animal systems, β-1,3-glucan has been reported to play an important role in immune-stimulatory activities. The related receptor of Dectin-1 has been cloned and the mechanisms are gradually elucidated (Brown et al., 2002). β-1,3-Glucan has also been proved as an elicitor to plant via the recognition as MAMPs or PAMPs. As a major component, Lam has been packaged as a commercial product in France in the past decades. However, β-1,3-glucan is still far away from fully understanding on the induced resistance in plants and the effects were always neglected in the fast defense responses. Therefore, we focused on the effects of β-1,3-glucan with different DP to signal transduction in tobacco. In this study, we had successfully established a chemical hydrolysis method to produce CRDO with β-anomeric configuration. Although enzymatic degradation of polysaccharide is always regarded as a green process to oligosaccharides, the procedure occurs with two stereochemical results: inversion or retention of the anomeric configuration at the position of cleavage. The outcomes produced by some "inverting" β-1,3-glucanase enzymes will possess α-anomeric configuration (Ferrer, 2006). Whether the stereochemical configuration change involves in the function of biological activities is still unknown. We showed that CRDO, Lam and COS triggered various defense and resistance responses in tobacco, including extracellular pH shift, H_2O_2 burst, NO release, the stomatal movement under light as well as protection against TMV.

The refractory state is well known as a convenient method to distinguish the different modes of perception (Binet, Bourque, Lebrun-Garcia, Chiltz, & Pugin, 1998; Felix, Regenass, & Boller, 1993; Menard et al., 2004; RouetMayer, Mathieu, Cazale, Guern,

& Lauriere, 1997). Tobacco cells initial treated with two sorts of β-1,3-glucan, respectively, were refractory states to a second stimulation of β-1,3-glucan, regardless of DP, which confirmed previous results just with Lam (Klarzynski et al., 2000; Menard et al., 2004). Conversely, CRDO was not refractory to the addition of COS. On the other hand, after pretreatment with COS, a second addition of CRDO or Lam, respectively, induced no refractory states of the pH shift of the extracellular medium. Moreover, CRDO still induced faster alkalinization of extracellular medium than Lam. Unlike many other elicitors, consecutive application of COS exhibited not only no refractory states but also remained a steady alkalinization of the suspension medium, which was in the similar way of β-megaspermin, a basic necrosis-inducing elicitin (Klarzynski et al., 2000). It was demonstrated that β-1,3-glucan and COS were recognized by different modes of perception in tobacco cells. It should be presumed that CRDO possessed the other perception systems far away from chemically sulfated Lam (Menard et al., 2004). These indicated different modes of perception to β-1,3-glucan and COS in tobacco cells and low DP oligosaccharides were more effective to alkalinization than high DP one at the initial period of the stimulation. CRDO and COS with low DP resulted in more rapid responses than Lam with high DP, illustrating that perhaps low DP exogenous carbohydrate elicitors were easily recognized by tobacco cells and took an active part in initial defenses. The various glucosidic bond and structure may be more important than the DP. Meanwhile, the differences of DP between CRDO and Lam led into recognition sequence.

H_2O_2 has been described as key roles in resistance responses against pathogens (Aziz et al., 2003; Menard, de Ruffray, Fritig, Yvin, & Kauffmann, 2005; Zhang et al., 2010). H_2O_2 is took account into involvement in phytoalexin production, lipid peroxidation and defense related genes expression, etc. (Jabs, Tschope, Colling, Hahlbrock, & Scheel, 1997; Rusterucci et al., 1996; Shinya et al., 2006). The method that high throughput screening of H_2O_2 burst from cells loaded with fluorescent probe in a 96 well cell plate offered a new avenue for fast screening useful elicitors and approximately online scrutinizing the H_2O_2 production. CRDO, Lam and COS all led to oxidative burst, while Lam and COS showed better effects than CRDO. It was demonstrated that skeleton of carbohydrate elicitors probably weighed greater than DP. Whether the role of H_2O_2 causes HR-like cell death gives rise to much controversy (Dorey, Kopp, Geoffroy, Fritig, & Kauffmann, 1999; Van Breusegem & Dat, 2006). Just as cell viability with trypan blue staining had insight into no HR by Lam (data not shown), which was in consistent with previously reported (Aziz et al., 2007; Klarzynski et al., 2000; Trouvelot et al., 2008), so was CRDO. Nevertheless, the positive results of COS were coincident with the results that apoptosis-like cell death elicited by COS was independent of H_2O_2 signal pathway (Wang, Li, Zhao, Du, & Lin, 2008).

In the past few decades, mounting evidences has been proved that NO is a vital signaling and defense molecule in mammals and plays a crucial role in activating disease resistances in plants, functioning as signaling molecular and maybe as an anti-microbial agent (Foissner, Wendehenne, Langebartels, & Durner, 2000). COS has been demonstrated to trigger the NO release from the epidermal peels, such as *Brassica napus* L. (Huyou 15) (Li et al., 2009) and tobacco (Zhao, She, Du, et al., 2007; Zhao, She, Yu, et al., 2007). To the best of our knowledge, it was the first time that β-1,3-glucan elicitors, CRDO and Lam, on the NO burst had been reported. L-NAME, one of the NOS inhibitors, effectively decreased the NO release triggered by three elicitors. Proposed mechanism had been postulated for three elicitors to generate NO mainly via NOS pathway. Both NO scavenger cPTIO and L-NAME reduced the chitosan protection against downy mildew disease (Manjunatha et al., 2009). The NO decrease alleviated the effects of elicitors for defense-inducing in *Arabidopsis* and tobacco (Delledonne, Xia, Dixon, & Lamb, 1998; Foissner et al., 2000; McDowell & Dangl, 2000). However, the specific pathway was still full of controversy.

Stomatal movement is recognized as one of the most sensitive responses to the biotic and abiotic stresses (Chen et al., 2004). Some plants have evolved a strategy to protect themselves against fungal attacks with a temporal and partial induction of stomatal movement. Nevertheless, few exogenous carbohydrate elicitors were reported to induce stomatal closure (Allegre et al., 2009; Lee et al., 1999; Li et al., 2009). Three elicitors discussed herein displayed the induction of stomatal closure and inhibition of opening, just like ABA. However, the capacities of elicitors could not keep pace with ABA. These results were in agreement with the previously stomatal movement on laminaran with DP 13 in grapevine (Allegre et al., 2009). Stomatal movement mainly resulted from H_2O_2 production (Davies, Bindschedler, Strickland, & Bolwell, 2006; Lamb & Dixon, 1997) and NO emission (Li et al., 2009) in guard cells, which implied CRDO and COS with low DP probably got easy access into quicker responses and higher level of oxidative burst than Lam with high DP.

The fact that the high DP glucan (Lam) had better protection than CRDO against TMV in tobacco maybe due to more ethylene-dependent basic PR protein 2 and 3 expressed by Lam. It was worth noting that the basic PR protein 5 could be induced by high DP instead of low one (Menard et al., 2004). Chitinase activity exhibited DP dependent mode in grapevine (Aziz et al., 2007). Although DP 5 was demonstrated to be the smallest elicitor-active structure in tobacco, the PAL activity induced by DP 5 was slight lower than by Lam (Klarzynski et al., 2000). Compared with β-1,3-glucan with low DP 3 or 6, high DP 10 triggered more expression of some defense genes at 20 h, such as *Chit4c*, *PGIP*, *PIN*, and so on (Aziz et al., 2007). However, previous reports focused on effects of single DP and durable defenses. Whether low DP glucan played vital roles in quick responses and resistance-inducing in plants has been unknown yet.

From the results mentioned above, we speculated that low DP oligosaccharides possibly functioned directly at the early stages. To some extent, they were likely to be easily recognized by plants, whereas elicitor with high DP had to be recognized by related receptors and then were degraded into small fragments to join the processes.

In order to elucidate the function of β-1,3-glucan with different DP on defense effects in tobacco. In the present study, low DP CRDO was used to compare with high DP Lam, while low DP COS as a positive control. We observed that all of them could act as elicitors to effectively cause defense and resistance-inducing reactions. Furthermore, although COS and CRDO had similar DP, defense effects were varied from each other. Low DP CRDO had a key role in the early events of plant resistance responses, such as alkalinization of extracellular medium and stomatal movement under light and so on. On the other hand, compared with CRDO, Lam with high DP had durable effects to activate the durable defense responses in tobacco against TMV. It should be noted that β-1,3-glucan with different DP played various roles in the resistance-inducing in tobacco. Oligosaccharides took an active part in the early defense reactions, whereas β-1,3-glucan with high DP possessed longer term defense effects.

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