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Inhibitory effects of sulfated lentinan with different degree of sulfation against tobacco mosaic virus (TMV) in tobacco seedlings

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ABSTRACT

The inhibitory effects of sulfated lentinan with different degrees of sulfation against tobacco mosaic virus (TMV) and the underlying mechanism were investigated. The results indicated that plants treated with increasing concentrations of sulfated lentinan, with increasing numbers of treatments and with increasing time after treatment had a decrease in the number of necrotic lesions, indicating a long-term protection against TMV that mimics vaccination. In addition, the levels of TMV-capsid protein (CP) transcripts decreased in distant leaves, indicating that sulfated lentinan induces systemic protection against TMV. The activities of the defense enzymes phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) and the amounts of several phenylpropanoid compounds (PPCs) were measured in control and treated plants without infection. A progressive increase in PAL activity was observed with increasing time after treatment, together with the accumulation of free and conjugated PPCs. In contrast, LOX activity remained unchanged. Interestingly, the increase in PAL activity showed a linear correlation with the decrease in necrotic lesions and the decrease in TMV-CP transcript level. Thus, sulfated lentinan induced systemic and long-term protection against TMV in tobacco plants that is determined, at least in part, by a sustained activation of PAL and the accumulation of PPCs with potential antiviral activity.

1. Introduction

Lentinan (LNT), which is derived from the fruiting body of *Lentinus edodes*, is a neutral polysaccharide and there are three single β-glucose branches randomly substituted at position 6 for every five main-chain D-glucose residues [1,2]. Lentinan has been shown to inhibit viral infections of both naked and enveloped viruses, besides antimicrobial and antibacterial activities [3]. Moreover, many studies reported that sulfated modification could enhance the antiviral activity of polysaccharides against avian infectious bronchitis virus, infectious bursal disease virus, dengue virus, herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus [4–7]. However, previous research on sulfated lentinan mainly focused on the effective way of sulfate modification, and on antioxidant, antitumor, antiviral and antiproliferative activities in vitro [3,8,9], the effect and mode of action of sulfated lentinan against tobacco mosaic virus (TMV) are largely unknown.

Sulfated polysaccharides have been proven as an elicitor to plants via the recognition as microbial- or pathogen-associated molecular patterns (PAMPs). These PAMPs can be recognized by pattern recognition receptors (PRRs) [10]. For example, sulfated laminarin, carrageenans and sulfated oligoglucuronans induced an enhanced protection against TMV [10–12]. Interestingly, their structures have a common backbone of β-(1, 3) linked glucopyranosyl residues but the polysaccharidic chain can be β-(1,6) branched with glucose or integrate some β-(1,4) linked glucopyranosyl residues in the main chain. Moreover, the biological activities of sulfated polysaccharides were shown to be dependent on their degree of substitution (DS) [13]. However, whether sulfated modification could improve the biological activity of LNT against TMV is not clear. Furthermore, the systemic sulfated LNT (sLNT) responses and the underlying mechanism of the sLNT-mediated disease resistance against TMV have not been elucidated. Therefore, we investigated the biological activities of the sulfated derivatives with different DS and its possible mechanisms in controlling TMV in tobacco seedlings.

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2. Materials and methods

2.1. Plant culture

Tobacco plants Xanthi^{NN} and Xanthi^{nn} were cultivated in flowerpots with a mixture of compost–vermiculite (3:2) in a growth chamber using a light/darkness period 16/8 h, at 22 ± 2 °C, 400 lx, and about 60 ± 5% relative humidity. All the seedlings that were used for experiment were healthy and have not been infected by any pathogens. Tobacco plants Xanthi^{nn} were used for TMV reproduction and to test suppression of TMV infection.

Tobacco mosaic virus (TMV) that came from our collection was multiplied in *Nicotiana tabacum*. TMV was extracted from infected leaves of systemically infected plants by homogenization in 0.05 M H_3PO_4 buffer (0.05 M KH_2PO_4, 0.05 M Na_2HPO_4 pH 5.5) with subsequent clarification of the extract by centrifugation at 2000 *g* for 6 min. The supernatant extract was used for mechanical inoculation.

2.2. Extraction and purification of LNT

L. edodes, bought from Fangge Company of Traditional Chinese Medicine, Zhejiang Province, was decocted with water into decoction. The crude total LNT (LNTtc) was extracted from the decoction by ethanol precipitation whose content was 70% in the decoction. LNTtc was purified as follows: to remove protein by Sevag's method [14], to remove pigment by active carbon adsorption, then through D101 macroaperture resin column, ADS_7 polymer adsorbents column, DEAE A_{25} cellulose and Sephadex G_{200} column in turn. The polysaccharide contents of LNTs were measured by Vitriol–anthrone taking anhydrous glucose as standard control. The polysaccharide contents of crude and purified LNT were 30.0% and 91.0%, respectively.

2.3. Sulfated modification of LNT

Purified LNT was sulfated by the chlorosulfonic acid–pyridine method and the modified conditions were based on the preparative experiment [15]. In brief: three chlorosulfonic acid–pyridine complex (1:6, 1:4, 1:2) were prepared in ice bath. Then, 400 mg LNT was added, respectively, stirred for 4 h at a temperature of 60 °C, dissolved in 100 mL ice-cold water, cooled to room temperature, neutralized with saturated NaOH solution and precipitated with 95% ethanol (EtOH). The sediments were re-dissolved with water. The solution was dialyzed against tap water for 48 h and distilled water for 12 h in turn, then, lyophilized to obtain three sLNTs, $sLNT_1$, $sLNT_2$ and $sLNT_3$. Their polysaccharide contents were 78.4%, 80.2% and 82.4%, respectively.

2.4. DS determination of sLNTs

The sulfur contents of three sLNTs were determined by Antonopoulos' method [4]. A calibration curve was constructed with sodium sulfate as standard. The degree of sulfation (DS) was calculated according to the equation: $DS = (1.62 \times S\%)/(32–1.02 \times S\%)$. The DSs of $sLNT_1$, $sLNT_2$ and $sLNT_3$ were 0.69, 0.98 and 1.37, respectively.

2.5. Plant treatment and disease evaluation

For protection assays against TMV infection, the whole tobacco plants ($n = 10$ for each group) at the 6–8 leaf stage with an initial height of 20 cm were sprayed with 1 mL LNT, $sLNT_1$, $sLNT_2$ and $sLNT_3$ solutions or distilled water (DW, control), at concentrations of 0, 25, 50 or 100 μg mL^{-1}, once a week for 3 weeks, and cultivated for 15 days after treatment. In addition, tobacco plants were treated with LNT, $sLNT_1$, $sLNT_2$, or $sLNT_3$ at a concentration of 100 μg mL^{-1}, once a week for 1–4 weeks, respectively, and cultivated for 15 days after treatment. Furthermore, tobacco plants were treated with LNT, $sLNT_1$, $sLNT_2$, or $sLNT_3$ at a concentration of 100 μg mL^{-1}, once a week for 3 weeks, and cultivated for 5, 15, 30, 45 or 60 days after treatment. Control and treated plants were infected with TMV in a single leaf and the number of necrotic lesions per leaf was counted after 5 days.

To quantify suppression of TMV infection, tobacco plants at the 6–8 leaf stage were infected with TMV in a single leaf located in the middle part of the plant, respectively. The tested plants were cultivated for 20 days after infection and then sprayed with distilled water (control) or LNT, $sLNT_1$, $sLNT_2$, or $sLNT_3$ at a concentration of 100 μg mL^{-1}, once a week for 3 weeks, and cultivated for 15 days after treatment. Apical leaves of the three control and treated plants were pooled in order to have triplicates to quantify TMV capsid protein (TMV-CP) transcripts.

For detection of enzyme activity and phenylpropanoid compounds, tobacco plants were sprayed in the upper and lower faces of all the leaves with distilled water (control) or LNT, $sLNT_1$, $sLNT_2$, or $sLNT_3$ at a concentration of 100 μg mL^{-1}, once a week, three times in total and cultivated for 45 days. Leaves were collected in the lower, middle and upper parts of each plant, pooled and frozen in liquid nitrogen.

2.6. Detection of viral transcripts by real-time RT-PCR

Total RNA was extracted from tobacco leaves (0.5 g of fresh weight) using FavorPrep Plant Total RNA kit (Favorgene, Ping Tung, Taiwan) and quantified with Quanti-iT Ribogreen RNA assay kit (Invitrogen, Oregon, USA). The relative level of transcripts coding for TMV-CP was determined using 18S RNA as internal control. PCR primers used to amplify TMV-CP transcripts were Forward-CP 5′-TGCCGAAACGTTAGATGCTACT-3′ and Reverse-CP 5′-TCCGGTTCCTCTGATCAATTCT-3′, those to amplify 18S RNA were Forward-18S 5′-TTCTTTGTACCTTTTGCTGGCTTAT-3′ and Reverse-18S 5′-CTCTGGTCCTTCTTTATACAACAAAC-3′. RT-PCR reactions were done using the Sensimix One-step kit (Quantace, London, UK) and real-time thermocycler Rotor gene 6000 (Corbett, Research, Sydney, Australia). RT-PCR reactions were performed using 5 μg of total RNA for TMV-CP and 18S RNA amplification, respectively, 10, and 0.4 μM of PCR primers for TMV-CP and 18S RNA amplification, respectively, and 2 mM $MgCl_2$. The reverse transcription step was done for 30 min at 49 °C and 42 °C for TMV-CP and 18SRNA amplification, respectively, and the inactivation step was performed for 10 min at 95 °C. PCR amplification reactions were done by 40 cycles at 95 °C for 20 s, using an annealing temperature and amplification time corresponding to 58 °C for 20 s for TMV-CP and 58 °C for 30 s for 18S RNA amplification and an elongation reaction of 30 s at 72 °C. Fragments amplified by RT-PCR were detected by fluorescence using SYBR GREEN I included in the amplification kit and the melting temperatures of amplified fragments correspond to 78.1 °C and 84.7 °C. RT-PCR reactions were done in triplicate from three independent replicates. Sample values were averaged, normalized using the ΔΔCT method and mean value control was subtracted from mean treated to determine fold of change in treated samples. The relative transcript level was expressed as $2^{-\Delta\Delta CT}$ [16].

2.7. Preparation of protein extracts

Frozen tobacco leaves (2 g) were homogenized and 6 mL 100 mM phosphate buffer pH 7.0 containing 5 mM β-mercaptoethanol and 20% (v/v) glycerol was added. After thawing and rehomogenization, the homogenate was filtered through Miracloth (Calbiochem, San Diego, CA). The filtrate was centrifuged for 15 min at 7400 × *g* and the supernatant was recovered and stored at −80 °C. Protein

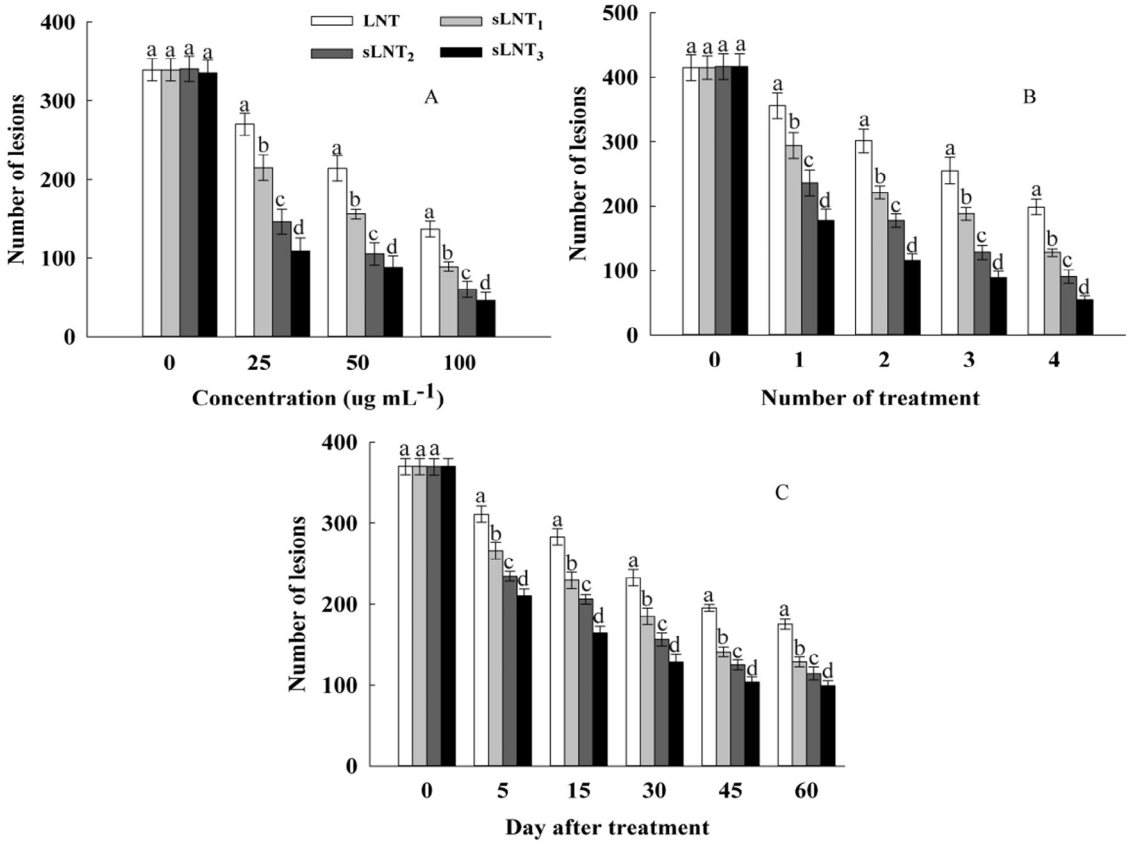

Fig. 1. Number of necrotic lesions per leaf in control tobacco plants and in plants treated with increasing concentrations of LNT, $sLNT_1$, $sLNT_2$ and $sLNT_3$ (A), with an increasing number of treatments (B) and cultivated for increasing times after treatment (C) and infected with tobacco mosaic virus. Bars correspond to mean values obtained from ten plants ± SD. Different letters indicate significant differences ($P < 0.05$).

concentrations were determined according to Bradford [17] and were normally 0.7 mg mL^{-1}.

2.8. Assay for PAL and LOX activities

The activities of defense-related enzymes were assessed in tobacco plants sprayed with sLNTs (10.0 µg mL^{-1}). The reaction mixture for PAL activity consisted of 1 mg mL^{-1} phenylalanine in 0.5 M sodium tetraborate buffer 0.1 M L^{-1} and boric acid buffer 0.1 M L^{-1} (pH 8.8) and 0.1 mL enzyme preparation. After 30 min of incubation at 40 °C, the reaction was stopped by adding 0.25 mL of 5 N HCl. The reaction mixture was cooled at 4 °C and 5 mL of distilled water was added. The amount of transcinnamic acid produced was measured spectrophotometrically at 290 nm. Phenylalanine ammonia-lyase activity was expressed as micrograms of transcinnamic acid per mg of protein per min [18]. LOX activity was determined in 1 mL of reaction mixture containing 100 mM phosphate buffer, pH 7.0, 0.4 mM linoleic acid and 200 µg of protein extract. The increase in absorbance caused by the accumulation of conjugated dienes was monitored at 234 nm for 10 min [19].

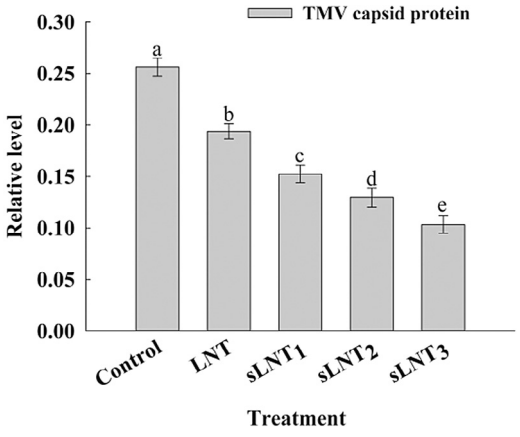

Fig. 2. Relative level of transcripts encoding TMV capsid protein in apical leaves of tobacco plants infected with TMV and treated with LNT, $sLNT_1$, $sLNT_2$ and $sLNT_3$, respectively. Bars correspond to mean values obtained from three pools of plants ± SD. Different letters indicate significant differences ($P < 0.05$).

2.9. Determination of total phenolic compounds

The levels of total phenolic compounds were determined using 0.2 g tobacco leaves (fresh weight), as described by Vera et al. [12]. Tobacco leaves were homogenized in 1 mL of ethanol 85% (v/v) using a plastic tube and pestle. The homogenate was centrifuged at 7500 *g* for 10 min using an Eppendorf microcentrifuge and the supernatant was recovered. A sample of 100 µL was mixed with 50 µL of Folin–Ciocalteau reagent (Merck, Darmstadt, Germany) and 700 µL of water and the solution was incubated for 1 h at room temperature. To stop the reaction 150 µL of 7% sodium carbonate was added and the absorbance was determined at 765 nm using a Genesys 5 spectrophotometer (Spectronic, Milton Roy Co., USA). Total phenolic compounds were expressed as microequivalents of gallic acid (Sigma, St. Louis, USA) using a calibration curve prepared with 0–50 µmol of gallic acid in a final volume of 1 mL.

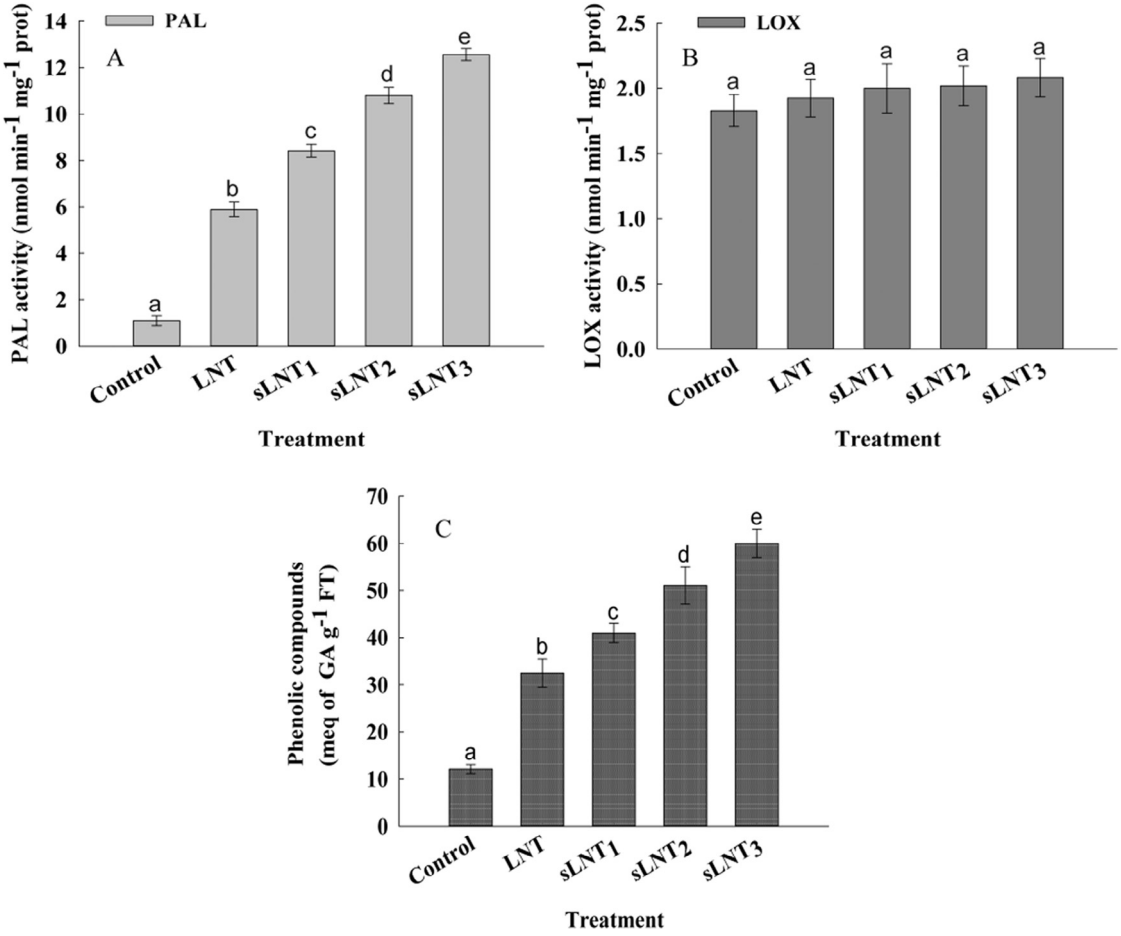

Fig. 3. Changes in PAL and LOX activity and total phenolic compound levels of tobacco plants treated with LNT, $sLNT_1$, $sLNT_2$ and $sLNT_3$, respectively. Bars correspond to mean values obtained from three pools of plants ± SD. Different letters indicate significant differences ($P < 0.05$).

3. Analysis of methanol-soluble phenylpropanoid compounds by HPLC

The levels of methanol-soluble phenylpropanoid compounds were determined using 1 g tobacco leaves (fresh weight), as described by Vera et al. [11]. Tobacco leaves were rapidly flash-frozen in liquid nitrogen and pulverized in a mortar with a pestle. Five milliliters of 100% methanol was added, the mixture was incubated in darkness for 24 h at room temperature, centrifuged at 7400 *g* for 15 min and the supernatant was recovered. An aliquot of 20 μL of methanol-soluble phenylpropanoid compounds was analyzed by HPLC using an Agilent equipment model 1110 (Agilent Technologies, Santa Cruz, USA), a reversed phase C-18 column (15.5 cm length, 4.6 mm inner diameter and 5 mm particle size) coupled to a photodiode array detector. Phenylpropanoid compounds were eluted with a linear gradient from 0 to 60% (v/v) of acetonitile in 5% (v/v) triphluoroacetic acid for 20 min and with a flow rate of 1 mL min^{-1}. Phenylpropanoid compounds were detected at 254, 280, 314 and 360 nm using absorption spectra of pure commercial standards (Sigma, St. Louis, USA) and quantified using a calibration curve prepared with pure standards at concentrations ranging from 0 to 1 mg mL^{-1}.

3.1. Statistical analysis

Each experiment was repeated three times. All statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA) according to Sokal and Rolph [20]. The significance of the observed differences was assessed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Differences between mean values were considered to be significant at a probability of 5% ($P < 0.05$).

4. Results

4.1. Sulfated lentinan induced suppression of viral infections against TMV

Tobacco plants treated with 25, 50 and 100 μg mL^{-1} of four glucans showed a decrease in the number of necrotic lesions corresponding to 20, 37 and 60% for LNT, to 36, 54 and 74% for $sLNT_1$, to 57, 69 and 82% for $sLNT_2$ and to 67, 73 and 86% for $sLNT_3$, respectively, compared with control plants (Fig. 1A). In addition, plants treated with 10.0 μg mL^{-1} four glucans, for one, two, three and four times, showed a decrease in the number of necrotic lesions corresponding to 14, 27, 38 and 56% for LNT, to 43, 51, 69 and 74% for $sLNT_1$, to 57, 79, 91 and 92% for $sLNT_2$, and to 39, 57, 69 and 79% for $sLNT_3$, respectively (Fig. 1B). Furthermore, plants treated with LNT and three sLNTs cultivated for 5, 15, 30, 45 and 60 days after treatment showed a decrease in the number of necrotic lesions corresponding to 16, 24, 37, 47 and 53% for LNT, to 28, 38, 50, 62 and 65% for $sLNT_1$, to 37, 44, 58, 66 and 69% for $sLNT_2$ and to 43, 56, 65, 72 and 74% for $sLNT_3$, respectively (Fig. 1C). In addition, the level of TMV-CP transcripts significantly decreased in plants treated with the four glucans LNT (19.8%), $sLNT_1$ (15.3%), $sLNT_2$ (13.2%) and $sLNT_3$ (10.3%),

Table 1
Levels of methanol-soluble phenylpropanoid compounds in tobacco plants treated with $sLNT_1$, $sLNT_2$ and $sLNT_3$.

Phenylpropanoid compound(s)[x]	Treatment	Level[y]	Increase ratio[z]
SA	Control	1.7 ± 0.3[a]	–
	LNT	2.3 ± 0.2[b]	1.4
	$sLNT_1$	2.5 ± 0.2[b]	1.5
	$sLNT_2$	3.2 ± 0.3[c]	1.9
	$sLNT_3$	3.9 ± 0.2[d]	2.3
SCO	Control	18.4 ± 6.7[a]	–
	LNT	73.6 ± 9.2[b]	4.0
	$sLNT_1$	102.3 ± 15.2[c]	5.6
	$sLNT_2$	139.7 ± 15.6[d]	7.6
	$sLNT_3$	178.5 ± 14.6[e]	9.7
RUT	Control	1.5 ± 0.6[a]	–
	LNT		
	$sLNT_1$	5.2 ± 0.2[b]	3.5
	$sLNT_2$	5.8 ± 0.3[b,c]	3.9
	$sLNT_3$	6.5 ± 0.4[c,d]	4.3
DHBA	Control	5.1 ± 0.2[a]	–
	LNT		
	$sLNT_1$	6.9 ± 0.3[b]	1.4
	$sLNT_2$	8.2 ± 0.5[c]	1.6
	$sLNT_3$	11.6 ± 0.6[d]	2.3
GA	Control	10.6 ± 2.6[a]	–
	LNT		
	$sLNT_1$	21.9 ± 3.5[b]	2.1
	$sLNT_2$	27.7 ± 3.7[b]	2.6
	$sLNT_3$	40.9 ± 4.4[c]	3.9
VA	Control	11.2 ± 1.5[a]	–
	LNT		
	$sLNT_1$	21.9 ± 2.5[b]	2.0
	$sLNT_2$	33.2 ± 2.4[c]	3.0
	$sLNT_3$	42.8 ± 1.4[d]	3.8
CHL	Control	2.6 ± 0.2[a]	–
	LNT		
	$sLNT_1$	4.5 ± 1.1[b]	1.7
	$sLNT_2$	6.1 ± 0.8[b,c]	2.3
	$sLNT_3$	7.5 ± 1.2[c,d]	2.9
CA	Control	11.2 ± 0.2[a]	–
	LNT		
	$sLNT_1$	42.9 ± 3.5[b]	3.8
	$sLNT_2$	51.3 ± 3.0[c]	4.6
	$sLNT_3$	60.5 ± 4.2[d]	5.4
ESC	Control	3.3 ± 0.2[a]	–
	$sLNT_1$	11.6 ± 0.9[b]	3.5
	$sLNT_2$	23.0 ± 2.2[c]	7.0
	$sLNT_3$	41.8 ± 3.5[d]	12.7
FA	Control	5.6 ± 0.8[a]	–
	LNT		
	$sLNT_1$	15.2 ± 2.2[b]	2.7
	$sLNT_2$	27.9 ± 2.4[c]	5.0
	$sLNT_3$	34.0 ± 4.5[c]	6.0
KAE	Control	0.2 ± 0.01[a]	–
	LNT		
	$sLNT_1$	0.9 ± 0.02[b]	4.5
	$sLNT_2$	1.1 ± 0.05[c]	5.5
	$sLNT_3$	1.5 ± 0.03[d]	7.5
QUE	Control	0.8 ± 0.1[a]	–
	LNT		
	$sLNT_1$	1.6 ± 0.2[b]	2.0
	$sLNT_2$	1.8 ± 0.1[b,c]	2.25
	$sLNT_3$	2.2 ± 0.2[c,d]	2.75

[x] SA, salycilic acid; SCO, scopoletin; RUT, rutin; DHBA, dehydroxybenzoic acid; GA, gallic acid; VA, vanillic acid; CHL, chlorogenic acid; CA, caffeic acid; ESC, esculetin; FE, ferulic acid; KAE, kaempferol; QUE, quercetin.

[y] The level of phenylpropanoid compounds is expressed as micrograms per gram of fresh tissue (FT) except CHL which is expressed as milligrams per gram of FT. Mean values ± SD were obtained from six different plants.

[z] Activation ratio = A1/A0, where A1 is the level of phenylpropanoid compounds of the treatment; A0 is the value of the control.

[a,b,c,d,e] Statistically significant difference compared with control, which was considered to be 100% ($P < 0.05$).

compared with the control plants (Fig. 2). Thus, LNT induced a dose-dependent, treatment number-dependent and long-term protection against TMV infection, especially sLNTs.

4.2. Changes in PAL and LOX activity and total phenolic compound levels

Significant increases in the level of PAL, LOX and total phenolic compounds were observed in plants treated with LNT and sLNTs, compared with that in the DW treatment ($P < 0.05$). Around 5.4, 1.4 and 2.7 times increases for PAL, LOX and total phenolic compounds were obtained in LNT treated leaves. Compared with LNT, the levels of PAL were about 1.4, 1.8, 2.1-fold higher for $sLNT_1$, $sLNT_2$ and $sLNT_3$, respectively, while the levels of LOX were 1.3, 1.5, 1.7-fold higher. In addition, the total phenolic compounds were about 1.3, 1.6, 1.8-fold higher for $sLNT_1$, $sLNT_2$ and $sLNT_3$, respectively, than that for the LNT treatment (Fig. 3).

4.3. Changes in methanol-soluble phenylpropanoid compound levels

The level of all tested phenylpropanoid compounds was significantly increased to varying degrees in treated plants, as compared to the control (Table 1). Moreover, significant differences were obtained between the LNT and sLNTs treatments. Additionally, the higher DS of sLNTs, the higher increases in phenylpropanoid compound levels. Thus, sLNTs induced a sustained increase in PAL activity and total phenolic compounds but a differential accumulation of phenylpropanoid compounds in tobacco plants.

5. Discussion

In the present study, sLNTs with different DS showed higher protective activities than LNT treatment against TMV in a dose-dependent, treatment number-dependent way, which were consistent with the previous studies that sulfated polysaccharides had a stronger protection against TMV than native ones [7,21] in a dose-dependent manner. These results also confirmed that sulfated modification could strengthen antiviral activity of LNT.

The biological activity of polysaccharides depends mainly on their molecular structure, especially when a certain chemical group is introduced, which often causes the changes in flexibility and spatial structure of the sugar chains thus resulting in changes of biological activity or making polysaccharides generate new activity [13]. The degree of sulfation (DS) is an index to reflect the modification degree. Much research confirmed that within a certain scope, the higher the DS, the better activity. Besides, the carbohydrate content is an important factor as well [22]. In this research, the $sLNT_3$ with the highest DS (1.37) and carbohydrate content (82.4%) presented the best activity, which was consistent with previous research.

The antiviral activity of sLNT and LNT in controlling TMV has been reported to involve a direct inactive property and elicitation effect on hosts [21]. The mechanism of direct antiviral activity of sulfated polysaccharide is considered that acid polyanion, e.g. SO_4^{2-}, can combine with the positive ions on the virus or cell surface, and thus stereo-inhibit viral adsorption and stop the virus to enter the cell or to replicate in the cell [5]. As an important role in virus replication, adsorption and mobility, the transcription of the TMV-CP gene in sLNT and LNT treated leaves decreased obviously in this study. Moreover, the relationship between the transcription level of the TMV-CP gene and DS of sLNT was an exact direct correlation. $sLNT_3$ with DS (1.37) had the highest inhibitory effect, followed by $sLNT_2$ (0.98) and $sLNT_1$ (0.69). However, whether the antiviral activities of LNT and sLNT were associated with the sulfate group affinity toward TMV CP needed further exploration.

With regard to the stimulated effect on hosts, sLNT activated the expression of several defense-related genes, such as

5-epi-aristolochene synthase, acidic chitinase class IV, phenylalanine ammonia-lyase, polygalacturonase inhibiting protein, stilbene synthase [23,24]. PAL is a key enzyme of the phenyl-propanoid pathway, contributing to the synthesis of phenolic compounds, phytoalexin and lignin, while LOX modulates the accumulation of jasmonates and oxylipins through the octadecanoid pathway [25–27]. In our research, increased transcript levels of PAL and LOX were induced and changed with the sulfated degree of sLNTs. In addition, the total phenolic compounds, which exhibit inhibitory activities against plant viruses [26,28] and are all associated with the process of local disease defense [25,29], were accumulated (Fig. 3). The reason that causes this phenomenon probably is a mutually synergistic interaction between the phenyl-propanoid and octadecanoid pathways. Such cross-talk provides the means by which plants can regulate their responses to maximize defense [30,31].

The elevated expression of defense genes is temporary [25], thus the long-term protection against TMV infection induced by sLNTs may reside in the ability of sulfated polysaccharides to stimulate the synthesis of compounds with antiviral activities. The synthesis of phenylpropanoid compounds (PPCs) and oxylipins with antimicrobial and antiviral activities was induced by sLNTs with the increased degree of sulfation, which is mainly regulated by the activity of phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) [32,33]. Interestingly, it has been shown that the accumulation of PPCs and other compounds having antiviral activities, such as benzoic acid, dihydroxybenzoic acid (DHBA), gallic acid (GA), caffeic acid (CA), chlorogenic acid (CHL), ferulic acid, scopoletin (SCO), esculetin (ESC), quercetin (QUE), kaempferol and rutin (RUT), terpenes, terpenoids and/or alkaloids, was observed in sulfated oligosaccharide treated plant leaves [11,12,34,35]. This indicates that the antiviral effect of sLNTs may reside, at least in part, in their ability to induce the activation of enzymes that synthesize secondary metabolites with antiviral activities.

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