The Effect of Chitosan and Chitin Oligomers on Gene Expression and Lignans Production in *Linum album* Cell Cultures

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Abstract

Background: ***Linum album*, a herbaceous and medicinal plant, has been shown to accumulate anti-tumor podophyllotoxin (PTOX) and related lignans.**

Objective: In this study, we have verified the time-course of cell growth, lignan production in cells treated with chitosan and chitin oligomers. To study mechanism of chitosan and chitin oligomers action, expression of phenylalanine ammonio-lyase (PAL), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD) and pinoresinol lariciresinol reductase (PLR) genes were investigated.

Methods: The cells were treated with 100 mg l^{-1} chitin and chitosan oligomers. Cells were harvested for 5 days after elicitation. lignans quantified by reverse-phase high-performance liquid chromatography (HPLC). Cell samples frozen with liquid N2 were used to elucidate the expression level of genes by quantitative PCR (qPCR).

Results: Cells elicited with chitosan hexamer for 5 days yielded the highest amount of PTOX (73.5 µg/g dry weight) and lariciresinol (96 µg/g DW) which were about 3-fold and 2-fold higher than control, respectively. The expression of genes by oligomers was increased, reaching a peak at 3 day after treatment.

Conclusion: Chitosan and chitin oligomers up-regulate the production of PTOX, by effecting on gene expression of PTOX biosynthesis pathway.

Keywords: ***Linum album*, Chitosan oligomers, Gene expression, Podophyllotoxin**

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Introduction

Linum album, an endemic species in Iran, is known to accumulate PTOX and its related lignans and has been targeted as a possible alternative source of PTOX [1, 2]. Podophyllotoxin (PTOX), with anti-tumor activity, is used as a precursor for the semi-synthesis of established cancer therapeutics such as etoposide, teniposide and etopophos [3]. The PTOX biosynthesis starts with the general phenylpropanoid pathway by activity of genes including phenylalanine ammonio-lyase (PAL), cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). Then pinoresinol lariciresinol reductase (PLR) regulates the lignans biosynthesis pathway. The positive effects of elicitors on the production of secondary metabolites have been verified [4, 5]. Elicitation may activate multiple genes responsible for plant defensive responses, leading to enhancement of secondary metabolite production [6]. Induced plant defensive responses involve a network of signal transduction such as reactive oxygen species (ROS) formation which is scaveraged by production of phenolic compounds [6]. Lignans as phenolic compounds are very well known to play an important role in plant defense [9]. Chitosan oligomers are known as elicitors of plant secondary metabolites production [10, 11]. We recently found novel elicitatory activities of fungal extracts and chitosan on lignan accumulation in *L. album* cell cultures [12, 13]. However, little is known about the expression profiles of genes in the lignan biosynthetic pathways upon elicitation. In this study, we have verified the time-course of lignan synthesis and expression of the lignan biosynthetic genes in *L. album* cell cultures treated with the chitosan and chitin oligomers.

Material and Methods

Cell suspension cultures

Cell suspension cultures of *Linum album* (Flax) were prepared from leaf explant-derived calli in 50 mL of Murashige and Skoog medium supplemented with 30 g L^{-1} sucrose, 2 mg L^{-1} NAA and 0.4 mg L^{-1} kinetin [12]. All suspension cultures were incubated on a gyratory shaker at 120 rpm in darkness at 25°C and subcultured every week.

Elicitation of *Linum album* cell cultures

For elicitation treatments, 500 mg of fresh cells were transferred to 5 ml of the cell culture medium per single well in a 6-well microliter plate, and were supplemented with 100 mg l^{-1} chitosan and chitin oligomers (Table 1) after 7 days of preculture (mid-log phase). The incubation continued on a shaker at 120 rpm in darkness at 25°C. Cells were collected for analyses at 1, 2, 3 and 5 d after elicitation.

Table 1- Full name and molecular weight (MW) of tested chitin and chitosan oligomers

Elicitors	Full name	Abbreviation	MW
Chitin pentamer	etra-*N*-Acetyl-chitotetraose	CHIT4	830
Chitin tetramer	Penta-*N*-Acetyl-chitopentaose	CHIT5	1034
Chitin hexamer	Hexa-*N*-Acetyl-Chitohexaose	CHIT6	1237
Chitosan tetramer	Chitoteraose hydrochloride	CH4	808
Chitosan pentamer	Chitopentaose hydrochloride	CH5	1006
Chitosan hexamer	Chitohexaose hydrochloride	CH6	1203

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Lignan quantification

Dried cells (50 mg) were homogenized in 1 mL of 80% methanol, followed by ethyl acetate extraction. The extract was dissolved in methanol again, and filtered through a Millex-LH filter with 0.45 μ pore size (Millipore, Bedford, MA, USA). Lignan quantification was analyzed using HPLC. The stationary phase was a C30-UG-5 column (Nomura Chemical, Japan). The elution solvent was composed of water and acetonitrile with a gradient system. This system with acetonitrile (A %) and water (B %) was used as follows: (0 min, flow rate 1): A (35), B (56); (10 min, flow rate 1): A (55), B (45); (38 min, flow rate 1): A (90), B (10); (55 min, flow rate 1): A (35), B (65). The presence of lignans in the samples was verified by mass spectrometry with commercially available standards [12].

Gene expression analyses

Cell samples frozen with liquid N2 were used to elucidate the expression level of phenylalanine ammonio-lyase (PAL), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD) and pinoresinol lariciresinol reductase (PLR) genes by quantitative PCR (qPCR). Total RNA was extracted using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) combined with DNase I treatment following the instructions provided. The quality and quantity of RNA were estimated using agarose gel electrophoresis and spectrophotometric measurements, respectively. Total RNA (1 μg), from each sample was reverse-transcribed by Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The primer sequences corresponding to the genes under study were designed using Gene Runner (Version 3.05) software (Table 2). Amplification was performed by Tag enzyme.

Table 2- Sequences of primers used to amplify the GADPH gene (used as a reference gene), phenylalanine ammonio-lyase (PAL), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD) and pinoresinol lariciresinol reductase (PLR) genes

Genes	Sequences	Amplicon size (bp)	Accession No.
GADPH	F- primer 5'- GGTGCCAAGAAGGTTGTCAT-3' R- primer 5'- TAACCTTAGCCAAGGGAGCA'-3'	148	AJ623267.1
PAL	F- primer 5'-GACGCTGCTGGGGCCTTCA-3' R- primer 5'-GGCGTCAAAAAGCACCATGGAG-3'	132	AY837828.1
CCR	F- primer 5'-CCTGTTGGAGCGACCTGGAGTTC-3' R- primer 5'-CCACCACGTCCACCTCTTTTTCC-3'	117	AJ440712.1
CAD	F- primer 5'-GGCCACATGGGAGTCAAAGTCG-3' R- primer 5'-GTCGGCGAGCTTCTGCATCTT C-3'	153	AJ811963.1
PLR	F- primer 5'-AGGAAGTATCCAGCGAAGCA-3' R- primer 5'-CACATTCGACGACAAAATGG -3'	96	AB525816.1

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Specificity of the primer for amplification was confirmed by melting-curve analysis performed by PCR, after 40 amplification cycles and gel-electrophoretic analysis. The PCR products were resolved in 2% (w/v) agarose gel. qPCR was performed using with the SYBR Green PCR Mastermix (Takara, Japan) and a cDNA dilution as a template in a 96-well platform system (ABI PRISM 7000 Sequence Detection System). The *L. album* GAPDH gene expression was used as an internal control. PCR amplification consisted of the initiation denaturing 95 °C for 10 s and 40 cycles of 5 s at 95 °C, annealing 30 s at 60 °C and extension 72 °C for 10 s. Data were analyzed using the 7000 system SDS software.

Statistical analysis

The results are presented as the mean ± standard error. Data were analyzed by one-way ANOVA with Dunnett error protection. Differences were accepted as significant for $p < 0.05$.

Results

Effect of elicitors on cell growth

The effects of elicitors on the growth of *L. album* cells were evaluated by measurement of the dry weight of the cells 5 days after treatment (Figure 1). Biomass of *L. album* cells was reduced significantly after 5 days by all of the elicitors. Chitosan hexamer (CH6) elicited the most potent growth inhibition where the biomass of *L. album* cells was reduced to 37% compared to the non-elicited control. The other oligomers decreased the growth of *L. album* cells by 14 – 33%.

Effect of exposure time of elicitors on lignan production

To clarify the functional correlation between lignan accumulation and the length of the elicitor treatment, we assessed the lignan content in *L. album* cells treated with chitosan and chitin oligomers for 1, 2, 3 and 5 days (Figures 2 and 3). The lignan contents were increased after elicitation by chitosan and chitin 5 days after treatment. However, this increase showed a general correlation with chain length and was generally higher for pentamer and hexamer forms than chitosan or chitin tetramers. The maximum total PTOX content was observed with treatments by CH6 (73.5 µg/g DW) and chitin hexamer (CHIT6) (61.3 µg/g DW) (Figure 2) which were about 3 fold more than control. Chitosan pentamer (CH5)- or chitin pentamer (CHIT)-treated plants showed 47% increase in PTOX content higher than control. Similarly, the cell cultures elicited by CH6 or CHIT6 for 5 days yielded the highest amount of lariciresinol which was about 2-fold higher than the non-elicited control cells (Figure 3).

Effect of fungal elicitors on gene expression

A rapid induction (d 1) of the PAL gene expression was observed in elicited cells with CH6, CH5 and CHIT5, CHIT6 persisted up to d 3 after elicitation (Figure 4a). PAL expression in the cells treated with CH6 reached to the peak at d 3, which was 4.4-fold higher than in the control, and decreased at d 5 but was still higher than control. When cells were treated with other oligomers, 1.8-fold significant induction of PAL gene expression was observed at d 3. The CCR gene expression in treated cells was not different from the control at d 1. However, it increased after 2 d for all treatments instead of CHIT4 and CH4. Among treatments, CH6 and CHIT6 showed the highest CCR gene expression after d 3 which was 1.5-fold higher than control (Figure 4b).

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The CAD gene expression reached a peak at d 3 and decreased at d 5 in all treatments (Figure 4c). As depicted in Figure 4c, the highest induction was observed in the cells elicited by CH6 at d 3 (1.9-fold) and decreased at d 5 but was still 1.8-fold higher than control.

The highest induction of the PLR gene expression was observed in the cells elicited by CH6 at d 3 (2.9-fold), compared to the control (Figure 4c). In addition, this PLR expression decreased at d 5.

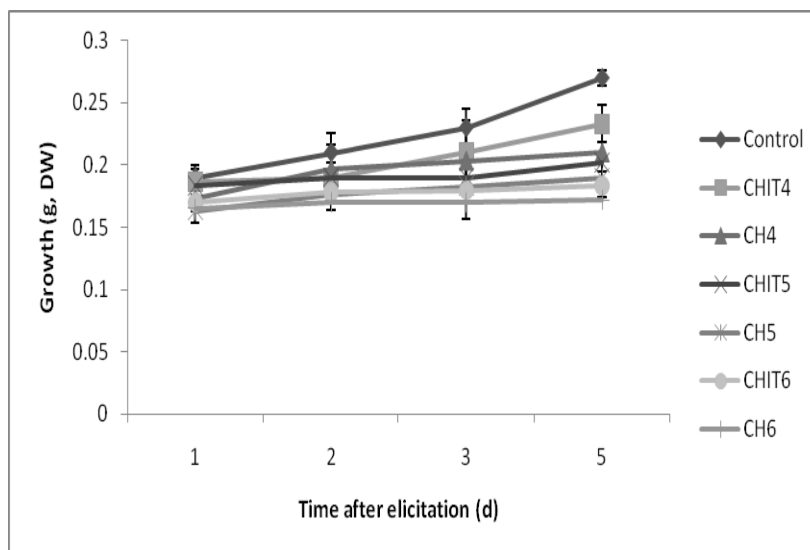

Figure 1- Time course of growth expressed as g of dry weight (D W) in control and chitosan and chitin-treated cultures. Oligomers were added after 7 d of growth and cultivated for further 5 d. Data represent average values from 3 separate experiments ± SD

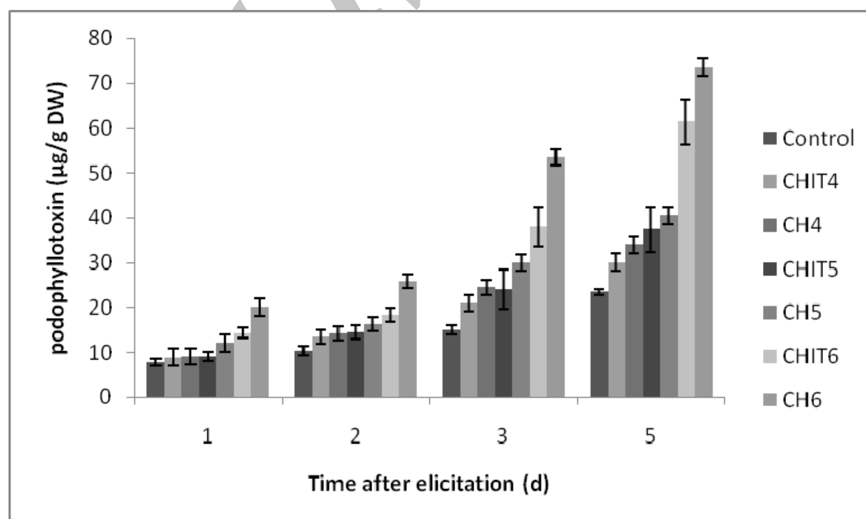

Figure 2- Time course of PTOX production in control and chitosan and chitin-treated cultures. Oligomers were added after 7 d of growth and cultivated for further 5 d. Data represent average values from 3 separate experiments ± SD

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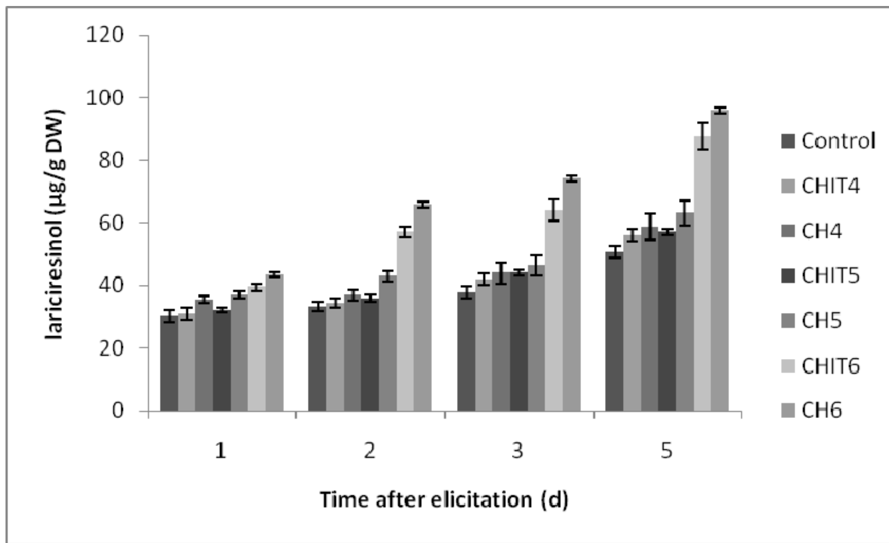

Figure 3- Chitosan and chitin-treated cultures. Oligomers were added after 7 d of growth and cultivated for further 5 d. Data represent average values from 3 separate experiments ± SD

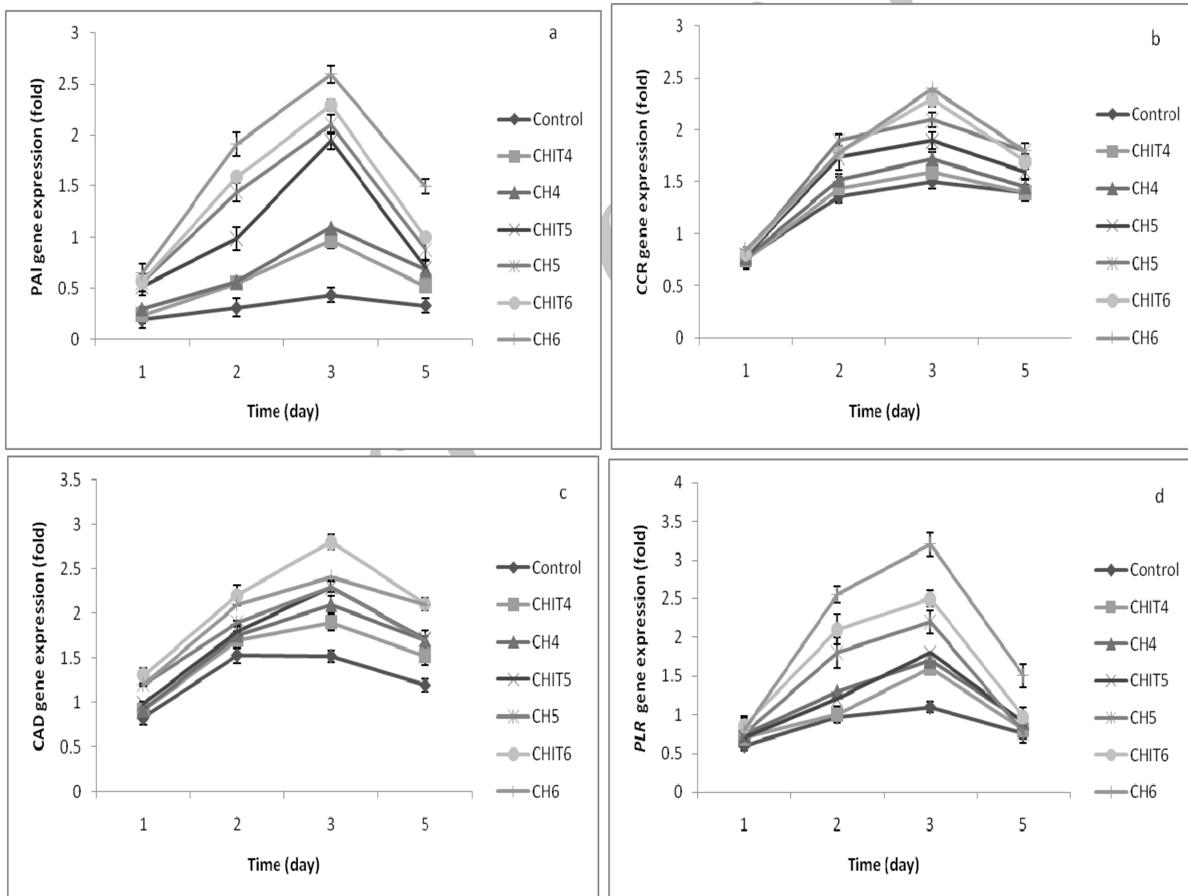

Figure 4- Expression level of the gene codifying for PAL (a), CCR (b), CAD (c) and PLR (d). Oligomers were added after 7 d of growth and cultivated for further 5 d. Data represent average values from 3 separate experiments ± SD

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The *PLR* gene expression in the cells treated with the CHIT6 and CH5 reached a peak at d 3, which was about 2-fold higher than control and decreased to the basal line at d 5.

The CH4, CHIT4 and CHIT5 induced gene expression of PLR at d 3 which were 1.5-fold higher than control (Figure 4d). The PLR gene expression induction decreased to the basal line at d 5.

Discussion

The present study has verified time courses in lignan accumulation and differences in relevant biosynthetic genes expression in cell suspension cultures of *L. album* by chitosan and chitin oligomers. We observed that in all chitosan and chitin treated cells, the lignans content was increased at d 5. This increase was generally dependent on the size of the chain of the oligomer; treatment with chitosan and chitin hexamers resulted in increased lignans content as compared to treatment with the tetramers. Cheng et al. [14] showed that the increase of phenylethanoid glycosides in *Cistanche deserticola* cell cultures depended on molecular weight of chitosan. Four genes, PAL, CCR, CAD and PLR, control important steps of the PTOX biosynthetic pathway [12], and were chosen to investigate the effect of treatments on the transcription. In this study, it was demonstrated that treatments with both chitin and chitosan oligomers caused the elevation of genes expression. Levels of genes expression were generally dependent on the chitosan or chitin chain length. The longer chains induced the greater level of gene expression. Khan et al. [10] also revealed that level of PAL activity in soybean leaves was dependent on the chitosan or chitin chain length. The present study showed that all treatment significantly up-regulated the PAL, CCR, CAD expression. These results suggested that all of the elicitors used in this study affected the early steps of PTOX production. In addition, the PLR gene expression is highly up-regulated at d 3 in the cells treated with the elicitors (Figure 4), in which the most abundant PTOX and lariciresinol were accumulated at d 5 (Figures 2 and 3), suggesting that PLR plays a vital role in the up-regulation of lignan production. Recently, we found functional correlation between increase in lignan production and up-regulation of PLR gene expression [15]. Comparing the time course of lignan production with the gene expression profile, a time lag was observed between the maximum gene expression and the maximum lignans contents (Figure 4). Similar metabolic profile has been reported [15, 16].

Conclusion

Taken as a whole, our results showed that treatment with chitosan and chitin increases lignan production, probably by modifying the expression pattern of key genes involved in the phenylpropanoid pathway and PLR gene responsible for the later step. Size of the chitosan and chitin oligomer chain exhibited a particularly variable effect on the accumulation of lignans and gene expression. These differences in the response to chitosan and chitin oligomers in *L. album* cell cultures suggest the existence of a complex control mechanism for lignans biosynthesis, which requires further studies.

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